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## Synthesis and Biological Evaluation of a Chitobiose-Based Peptide N-Glycanase Inhibitor Library

Witte, Martin D.; Horst, Danielle; Wiertz, Emmanuel J.H.J.; Marel, Gijsbert A. van der; Overkleeft, Herman S.

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# Supporting Information

## Synthesis and biological evaluation of a chitobiose based peptide *N*-glycanase inhibitor library

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### Table of contents

General Procedures	S4
Synthesis of <b>5</b>	S5
Synthesis of <b>7</b>	S6
Synthesis of <b>26</b>	S6
Synthesis of <b>39</b>	S7
Synthesis of <b>49</b>	S8
Competition assay	S9
In vivo inhibition of human PNGase	S9
Copy of <sup>1</sup> H-NMR of <b>5</b>	S11

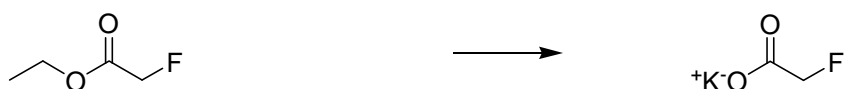
Copy of $^{13}\text{C}$ -NMR of <b>5</b>	S12
Copy of $^1\text{H}$ -NMR of <b>7</b>	S13
Copy of $^{13}\text{C}$ -NMR of <b>7</b>	S14
Copy of $^1\text{H}$ -NMR of <b>10</b>	S15
Copy of $^{13}\text{C}$ -NMR of <b>10</b>	S16
Copy of LC/MS spectrum of <b>10</b>	S17
Copy of $^1\text{H}$ -NMR of <b>11</b>	S18
Copy of $^{13}\text{C}$ -NMR of <b>11</b>	S19
Copy of LC/MS spectrum of <b>11</b>	S20
Copy of $^1\text{H}$ -NMR of <b>12</b>	S21
Copy of $^{13}\text{C}$ -NMR of <b>12</b>	S22
Copy of LC/MS spectrum of <b>12</b>	S23
Copy of $^1\text{H}$ -NMR of <b>13</b>	S24
Copy of $^{13}\text{C}$ -NMR of <b>13</b>	S25
Copy of LC/MS spectrum of <b>13</b>	S26
Copy of $^1\text{H}$ -NMR of <b>14</b>	S27
Copy of $^{13}\text{C}$ -NMR of <b>14</b>	S28
Copy of LC/MS spectrum of <b>14</b>	S29
Copy of $^1\text{H}$ -NMR of <b>17</b>	S30
Copy of $^{13}\text{C}$ -NMR of <b>17</b>	S31
Copy of LC/MS spectrum of <b>17</b>	S32
Copy of $^1\text{H}$ -NMR of <b>18</b>	S33
Copy of $^{13}\text{C}$ -NMR of <b>18</b>	S34

Copy of LC/MS spectrum of <b>18</b>	S35
Copy of $^1\text{H}$ -NMR of <b>19</b>	S36
Copy of $^{13}\text{C}$ -NMR of <b>19</b>	S37
Copy of LC/MS spectrum of <b>19</b>	S38
Copy of $^1\text{H}$ -NMR of <b>20</b>	S39
Copy of $^{13}\text{C}$ -NMR of <b>20</b>	S40
Copy of LC/MS spectrum of <b>20</b>	S41
Copy of $^1\text{H}$ -NMR of <b>21</b>	S42
Copy of $^{13}\text{C}$ -NMR of <b>21</b>	S43
Copy of LC/MS spectrum of <b>21</b>	S44
Copy of $^1\text{H}$ -NMR of <b>24</b>	S45
Copy of $^{13}\text{C}$ -NMR of <b>24</b>	S46
Copy of LC/MS spectrum of <b>24</b>	S47
Copy of $^1\text{H}$ -NMR of <b>25</b>	S48
Copy of $^{13}\text{C}$ -NMR of <b>25</b>	S49
Copy of LC/MS spectrum of <b>25</b>	S50

## General Procedures:

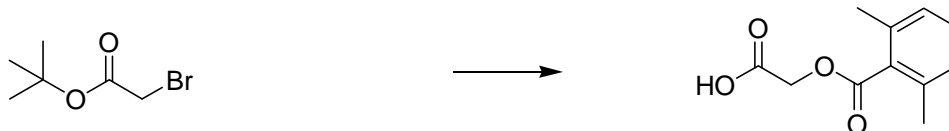
All reagents were commercial grade and were used as received unless stated otherwise. TTBP was synthesized as described. Diethyl ether ( $\text{Et}_2\text{O}$ ), ethyl acetate ( $\text{EtOAc}$ ), light petroleum ether (PE) and toluene (Tol) were purchased from Riedel-de Haën. Acetonitrile (MeCN), dichloroethane, dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), *N,N*-dimethylformamide (DMF), methanol (MeOH), pyridine (pyr), tetrahydrofuran (THF) were obtained from Biosolve. THF was distilled over  $\text{LiAlH}_4$  prior to use. Before use  $\text{CH}_2\text{Cl}_2$  was refluxed over  $\text{CaH}_2$  for 2h and distilled. Trace of water were removed by refluxing *n*-Butanol over sodium for 2h followed by distilling and storing over 3Å MS. Trifluoromethanesulfonic anhydride was distilled from  $\text{P}_2\text{O}_5$ . Molecular sieves 3Å were flame dried before use. All reactions were performed under an inert atmosphere of Argon unless stated otherwise. Solvents used for flash chromatography were of pro analysi quality. Flash chromatography was performed on Screening Devices silica gel 60 (0.04 – 0.063 mm). TLC-analysis was conducted on DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm) were applicable and by spraying with 20% sulfuric acid in ethanol followed by charring at  $\sim 150^\circ\text{C}$  or by spraying with a solution of  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot\text{H}_2\text{O}$  (25 g/l) and  $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4\cdot 2\text{H}_2\text{O}$  (10g/l) in 10% sulfuric acid in water followed by charring at  $\sim 150^\circ\text{C}$ .  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a 400 400/100 MHz, 500/125 MHz and a 600/150 MHz spectrometer. Chemical shifts ( $\delta$ ) are given in ppm relative to the chloroform residual solvent peak or tetramethylsilane as internal standard. Coupling constants are given in Hz. All given  $^{13}\text{C}$  spectra are proton decoupled. High resolution mass spectra were recorded by direct injection (2  $\mu\text{L}$  of a 2  $\mu\text{M}$  solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass

spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution  $R = 60000$  at  $m/z$  400 (mass range  $m/z = 150$ -2000) and dioctylphthalate ( $m/z = 391.28428$ ) as a “lock mass”. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on a HPLC system with a standard  $C_{18}$  (4.6 mmD  $\times$  250 mmL, 5 $\mu$  particle size) column (detection at 200-600 nm) in combination with buffers A:  $H_2O$ , B: MeCN and C: 0.5% aq. TFA and coupled to a mass spectrometer with ESI. For RP-HPLC purifications an automated HPLC system equipped with a  $C_{18}$  (5 $\mu$ m 250 $\times$ 10 mm) column was used. The applied buffers were A: 0.1% TFA in  $H_2O$ , B: MeCN.



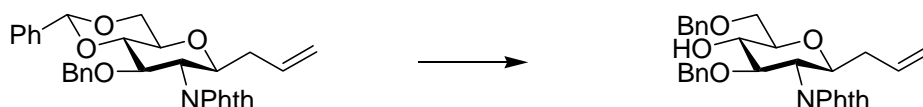
### Potassium fluoroacetate (**5**)

Ethyl fluoroacetate, was synthesized as described in literature.<sup>1</sup> Ethyl fluoroacetate (0.107 g, 1 mmol) was dissolved in absolute EtOH, cooled to 0°C and potassium hydroxide (56.1 mg, 1 mmol) was added. The reaction was stirred overnight after which petroleum ether was added. The solution was cooled to -20°C and filtered giving title compound **5** (66%, 77 mg, 0.66 mmol) as a white solid. <sup>1</sup>H-NMR (400 MHz, D<sub>2</sub>O)  $\delta$  4.75 (d,  $J = 48.3$ , 2H). <sup>13</sup>C-NMR (101 MHz, D<sub>2</sub>O)  $\delta$  ppm 79.5 (d,  $J = 176$ ). FT-IR:  $\nu_{max}$  (neat)/cm<sup>-1</sup> 3381, 1606, 1423, 1398, 1337, 1009. Mp: 189°C (start of decomposition).



### 2-(2,6-dimethylbenzoyloxy)acetic acid (7)

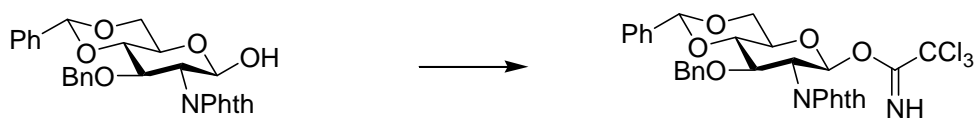
*Tert*-butyl bromoacetate (0.437 ml, 3 mmol), dimethylbenzoic acid (0.496 g, 3.3 mmol) and potassium fluoride (0.261 g, 4.5 mmol) were dissolved in DMF. After overnight stirring, Et<sub>2</sub>O was added after which the solution was extracted with NaHCO<sub>3</sub> (sat. aq.). The organic layer was dried, concentrated *in vacuo* and subsequently dissolved in TFA/H<sub>2</sub>O (95/5). The solution was concentrated after 1h, coevaporated with toluene to remove residual traces of TFA and applied to silica gel chromatography (0→10% EA/Tol) giving title compound **7** (81%, 0.506 g, 2.43 mmol) as a white solid. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 7.22 (dd, *J* = 15.2 Hz, 7.6 Hz, 1H), 7.05 (d, *J* = 7.6 Hz), 4.89 (s, 2H), 2.37 (s, 6H). <sup>13</sup>C-NMR (101 MHz, CDCl<sub>3</sub>) δ ppm 173.7, 169.1, 135.7, 132.1, 129.9, 127.7, 60.4, 19.8. FT-IR:  $\nu_{max}$  (neat)/cm<sup>-1</sup> 2955.7, 1720.7, 1597.4, 1462.9, 1424.3, 1306.1, 1281.6, 1240.1, 1165.5, 1113.7, 1082.7, 1029.1, 1003.3. Mp: 128.7-130.4°C.



### 3-C-(3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl) propene (26)

Known                      3-*C*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-propene<sup>2</sup> (0.512 g, 1 mmol) was coevaporated with toluene (3×) before being dissolved in freshly distilled CH<sub>2</sub>Cl<sub>2</sub>. Activated molsieves (4 Å) and triethylsilane (0.533 mL, 3.3 mmol) were added. The mixture was stirred at room temperature for 15 min, cooled to -78°C followed by the addition of

trifluoromethanesulfonic acid (0.265 mL, 3 mmol). TLC-analysis showed complete conversion after 45 min after which the reaction was quenched with MeOH/Et<sub>3</sub>N, extracted with NaHCO<sub>3</sub> and concentrated. Silica gel purification (0→30% EA/PE) afforded acceptor **26** (70%, 0.359 g, 0.70 mmol) as an oil. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 7.80-7.75 (m, 1H), 7.70-7.63 (m, 3H), 7.38-7.23 (m, 5H), 7.05-6.98 (m, 2H), 6.95-6.90 (m, 3H), 5.72 (dt, *J* = 16.9, 16.8, 7.0 Hz, 1H), 4.89 (d, *J* = 17.2 Hz, 1H), 4.83 (d, *J* = 10.2 Hz, 1H), 4.76 (d, *J* = 12.2 Hz, 1H), 4.64 (d, *J* = 12.1 Hz, 1H), 4.58 (d, *J* = 12.1 Hz, 1H), 4.52 (d, *J* = 12.2 Hz, 1H), 4.30-4.18 (m, 2H), 4.08 (t, *J* = 10.0, 10.0 Hz, 1H), 3.82-3.71 (m, 3H), 3.61 (td, *J* = 9.1, 4.4, 4.4 Hz, 1H), 3.04 (s, 1H), 2.27-2.13 (m, 2H). <sup>13</sup>C-NMR (101 MHz, CDCl<sub>3</sub>) δ ppm 168.1, 167.8, 138.2, 137.7, 133.8, 133.7, 133.3, 131.5, 131.4, 128.4, 128.0, 127.70, 127.65, 127.2, 123.2, 123.1, 116.9, 79.6, 77.6, 74.4, 74.2, 73.6, 70.6, 55.3, 36.9. FT-IR:  $\nu_{max}$  (neat)/cm<sup>-1</sup> 3473.9, 2869.8, 1774.6, 1709.7, 1383.9, 1201.4, 1076.3, 913.3. ESI/MS: *m/z* = 514.13 (M+H)<sup>+</sup>. HRMS: (M + Na<sup>+</sup>) calc. for C<sub>31</sub>H<sub>31</sub>NO<sub>6</sub> 536.20436, found 536.20397.



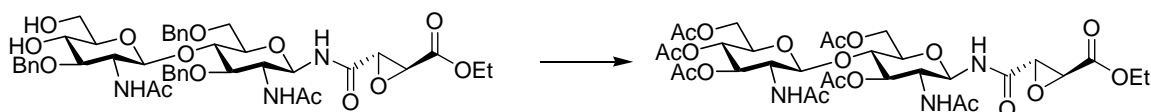
### Imidate (**39**)

3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranose<sup>3</sup> (2.88 g, 5.9 mmol) was dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub>. K<sub>2</sub>CO<sub>3</sub> (3.017 g, 21.83 mmol) and trichloroacetonitrile (2.97 mL, 29.5 mmol) were added. After overnight stirring, the mixture was filtered, concentrated in vacuo and purified by silicagel column chromatography. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 8.58 (s, 1H), 7.78-7.66 (m, 4H),



7.55-7.51 (m, 2H), 7.44-7.37 (m, 3H), 7.03-6.99 (m, 2H), 6.95-6.86 (m, 3H), 6.50 (d,  $J = 8.3$  Hz, 1H), 5.64 (s, 1H), 4.82 (d,  $J = 12.4$  Hz, 1H), 4.60-4.45 (m, 4H), 3.94-3.80 (m, 3H).  $^{13}\text{C}$ -NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  ppm 167.4, 160.8, 137.7, 137.1, 134.0, 131.4, 129.1, 128.3, 128.0, 127.4, 126.1, 123.4, 101.4, 94.3, 82.6, 74.4, 74.2, 68.5, 66.9, 54.7. FT-IR:  $\nu_{\text{max}}$  (neat)/ $\text{cm}^{-1}$  1776.4, 1714.1, 1677.8, 1453.7, 1383.8, 1294.2, 1112.9, 1053.4, 1023.1.

The imidate was hydrolyzed during mass-spectrometry. HRMS: (M-imidate +  $\text{Na}^+$ ) calc. for  $\text{C}_{31}\text{H}_{31}\text{NO}_6$  510.15232, found 510.15212.



**(2*S*,3*S*)-3-*N*-(*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- $\beta$ -D-glucopyranosyl)-(1 $\rightarrow$ 4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy- $\beta$ -D-glucopyranosylcarbamoyl) oxirane-2-carboxylic acid ethyl ester (**49**)**

Known epoxide<sup>2</sup> (166 mg, 0.199 mmol) was dissolved in EtOH/Dioxane (2/1 v/v, 5 mL).  $\text{Pd}(\text{OH})_2$  on charcoal (25 mg) was added followed by stirring under  $\text{H}_2$  atm for 1h. The solution was filtered, concentrated, redissolved in pyridine (5 mL) and cooled to  $0^\circ\text{C}$  before being treated with  $\text{Ac}_2\text{O}$  (3 mL). The mixture was stirred overnight, concentrated, redissolved in  $\text{CH}_2\text{Cl}_2$ , washed with  $\text{H}_2\text{O}$ , dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated in vacuo. RP-HPLC (linear gradient 22  $\rightarrow$  32% B in 10.00 min) gave title compound **49** (26%, 39.69 mg, 51.2  $\mu\text{mol}$ ) as a white solid.  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3/\text{MeOD}$ )  $\delta$  ppm 5.27 (dd,  $J = 10.2, 9.5$  Hz, 1H), 5.09 (dd,  $J = 10.5, 8.4$  Hz, 1H), 5.07-4.98 (m, 2H), 4.67 (d,  $J = 8.3$  Hz, 1H), 4.43 (dd,  $J = 12.1, 1.4$  Hz, 1H), 4.42 (dd,  $J = 12.4, 4.4$  Hz, 1H), 4.28 (m, 2H), 4.07 (dd,  $J = 11.9, 4.7$  Hz, 1H), 4.05-3.98 (m, 2H), 3.78-3.69 (m, 4H), 3.62 (d,  $J = 1.7$

Hz, 1H), 3.48 (d,  $J = 1.7$  Hz, 1H), 2.14 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.02 (s, 6H), 1.94 (s, 3H), 1.91 (s, 3H), 1.32 (t,  $J = 7.1, 7.1$  Hz, 3H).  $^{13}\text{C}$ -NMR (101 MHz,  $\text{CDCl}_3/\text{MeOD}$ )  $\delta$  ppm 172.3, 171.6, 171.2, 170.7, 170.6, 170.5, 169.6, 167.0, 166.6, 100.2, 78.2, 75.2, 74.2, 72.6, 71.9, 71.2, 68.1, 62.14, 62.09, 61.5, 54.4, 52.9, 52.4, 51.8, 22.11, 22.08, 20.3, 20.2, 20.1, 20.04, 19.97, 13.5. FT-IR:  $\nu_{\text{max}}$ (neat)/ $\text{cm}^{-1}$  3288.1, 1738.9, 1665.5, 1541.9, 1435.1, 1372.0, 1228.2, 1046.1, 902.1, 600.8. LC/MS:  $R_t$  4.700 min; linear gradient 10 $\rightarrow$ 90% B in 13.5 min; ESI/MS:  $m/z = 776.5$  ( $\text{M}+\text{H}$ ) $^+$ . HRMS: ( $\text{M} + \text{H}^+$ ) calc. for  $\text{C}_{32}\text{H}_{45}\text{N}_3\text{O}_{19}$  776.27200, found 776.27207.

### Competition assay

To 9  $\mu\text{L}$  of a solution of yeast PNGase (11.1 ng/ $\mu\text{L}$ ) in PBS (pH = 7.2, 20 mM sodium phosphate, 150 mM NaCl, BSA (1  $\mu\text{g}/\mu\text{L}$ ) was added inhibitor (1  $\mu\text{L}$ ) and Bodipy-VAD-Fmk (1  $\mu\text{L}$ , 0.5  $\mu\text{M}$  final concentration). The resulting solution was incubated at 37°C for 1h, after which it was quenched by the addition of 4  $\times$  SDS-PAGE sample buffer (4  $\mu\text{L}$ ) and boiling for 5 min. The proteins were separated on a 10% SDS-PAGE gel and subsequently the fluorescence was measured in the wet gel slabs by using the CY3/Tamra settings ( $\lambda_{\text{ex}}$  532  $\lambda_{\text{em}}$  560) on a Typhoon Variable Mode Imager (Amersham Biosciences). The remaining fluorescence was quantified with ImageQuant followed by analysis with Graphpad Prism.

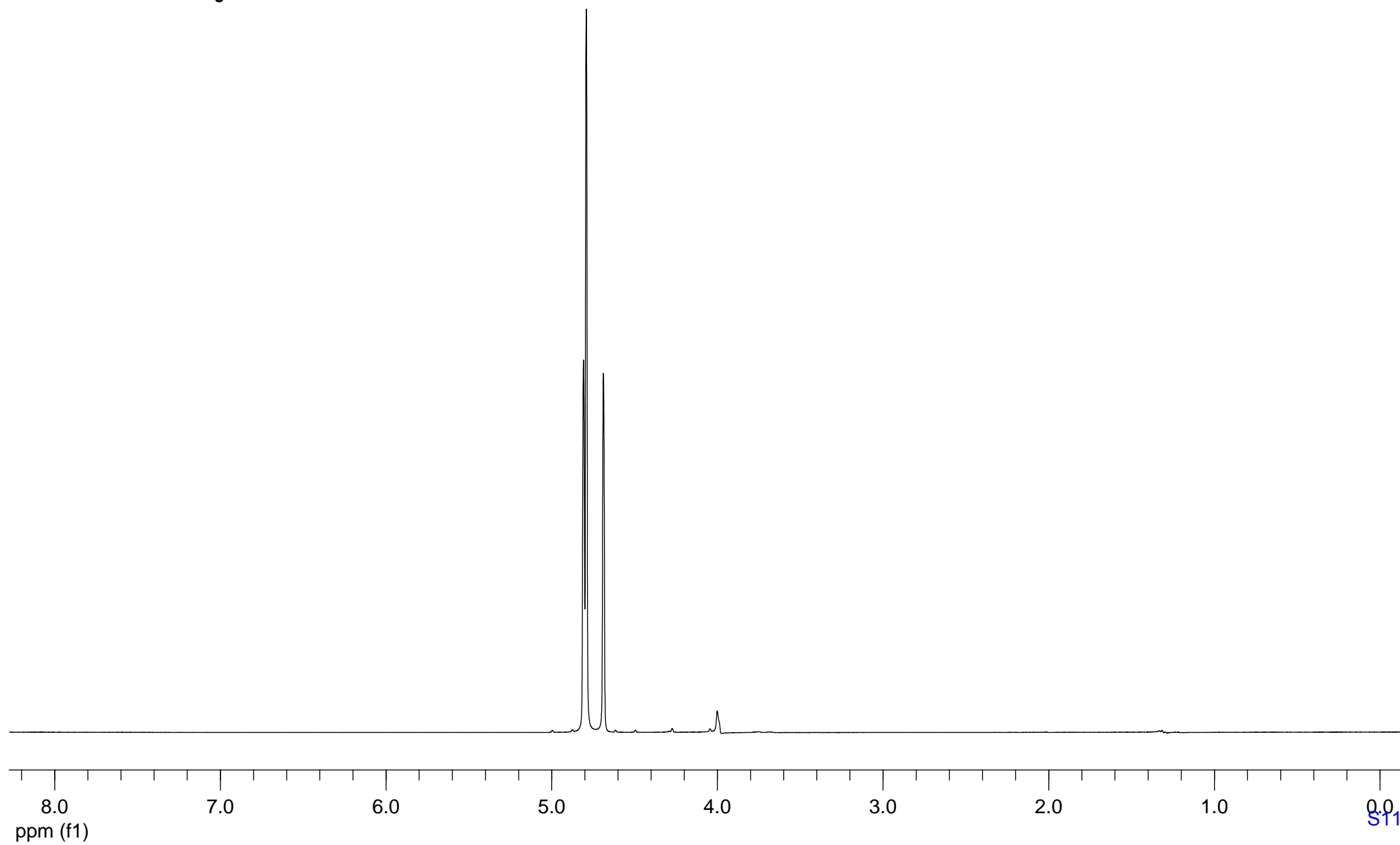
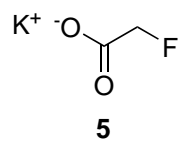
### In vivo inhibition of human PNGase

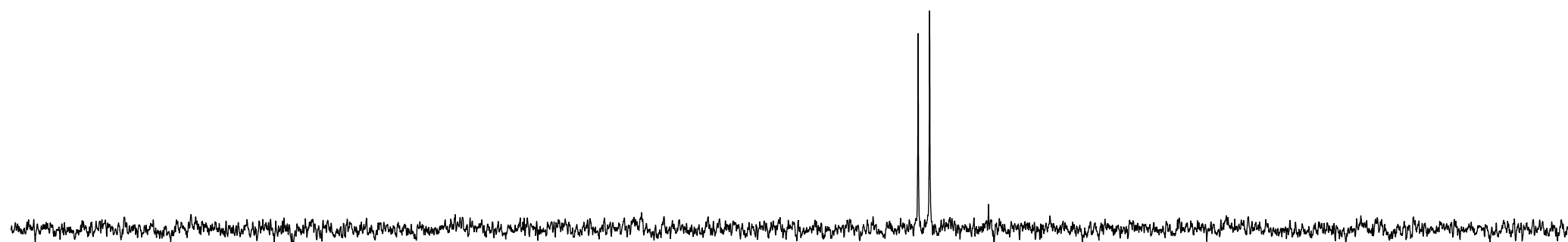
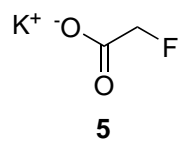
Control and US11-expressing U373 cells ( $1.5 \times 10^6$  cells per lane) were collected, washed with PBS and resuspended in Met $^-$ /Cys $^-$  medium (2 mL) containing 10% FCS and the

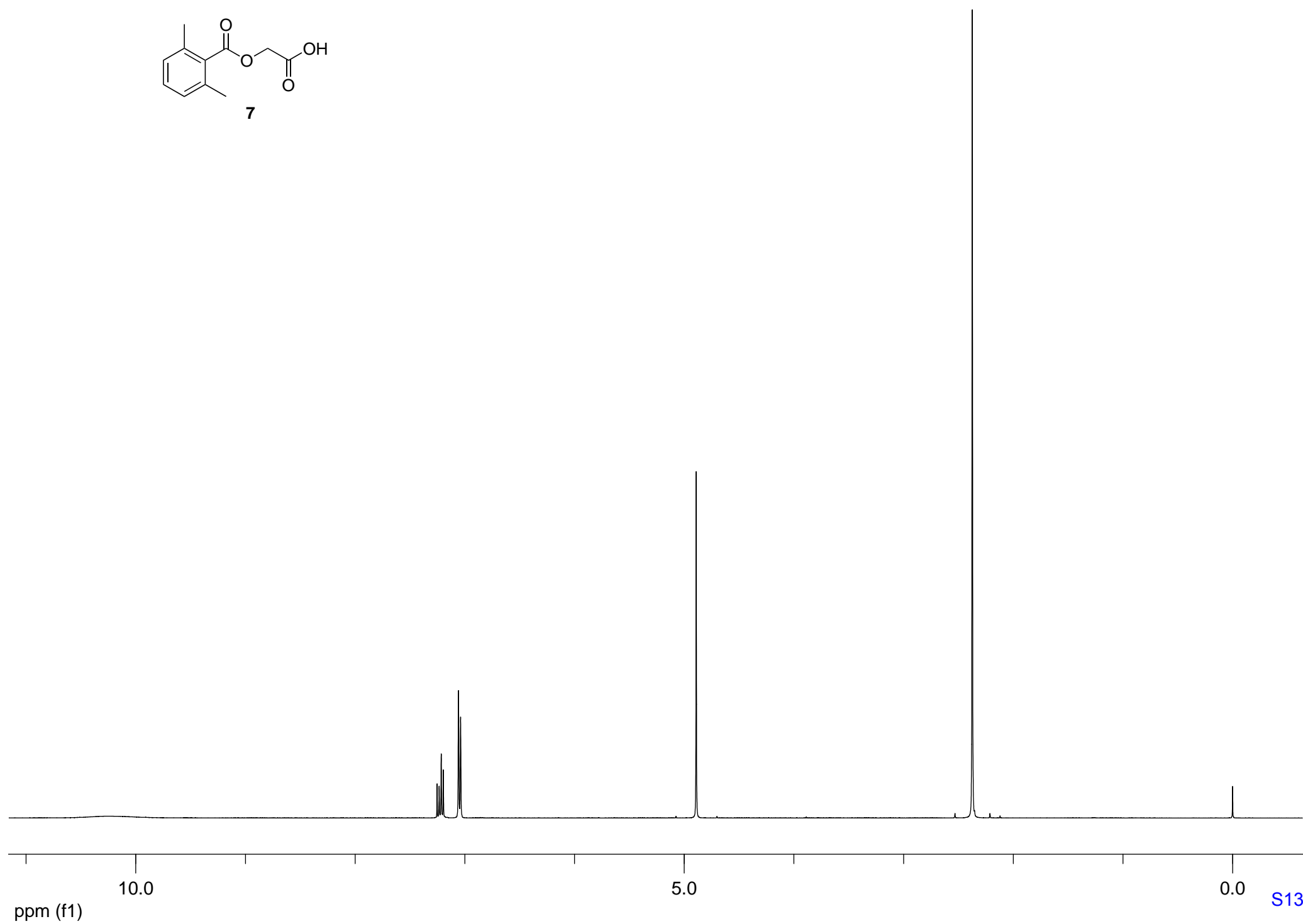
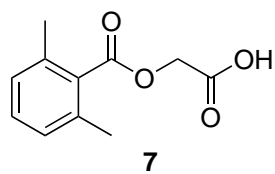
inhibitors ZL<sub>3</sub>VS (1.25  $\mu$ M), ZVAD-Fmk, **2**, **49** (30  $\mu$ M and 100  $\mu$ M respectively) and NGT (50  $\mu$ M) as indicated. The cells were incubated for 45 min at 37°C, pelleted by centrifugation and resuspended in 250  $\mu$ L Met<sup>-</sup>/Cys<sup>-</sup> medium, again in the presence of the inhibitors. [<sup>35</sup>S]-labeled Cys/Met (5  $\mu$ L, 200  $\mu$ Ci/mL) was added and the cells were incubated for 30 minutes at 37°C. The cells were washed with PBS and subsequently lysed in 1 ml cold NP-40 buffer (0.5% NP-40, 50 mM Tris HCl pH 7.5, 5 mM MgCl<sub>2</sub>, 10  $\mu$ M leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) for 45 min at 4°C. The lysed cells were centrifuged (16.000 g, 15 min) at 4°C in order to obtain post-nuclear lysates. These lysates were precleared twice by the addition of Protein G- and Protein A-sepharose beads (7.5  $\mu$ L, GE Healthcare), normal rabbit serum (1.5  $\mu$ L) and normal mouse serum (1.5  $\mu$ L), followed by incubation for 60 min at 4°C. Next, the class I heavy chains were immunoprecipitated. Specific antibodies (mAb HC10), Protein G- and Protein A-sepharose beads were added to the lysates and samples were incubated o/n at 4°C. Isolated immune complexes were washed thrice with NET buffer containing 0.1% SDS and denatured in reducing sample buffer (50  $\mu$ L). The samples were separated by SDS-PAGE and visualized by phosphoimaging.

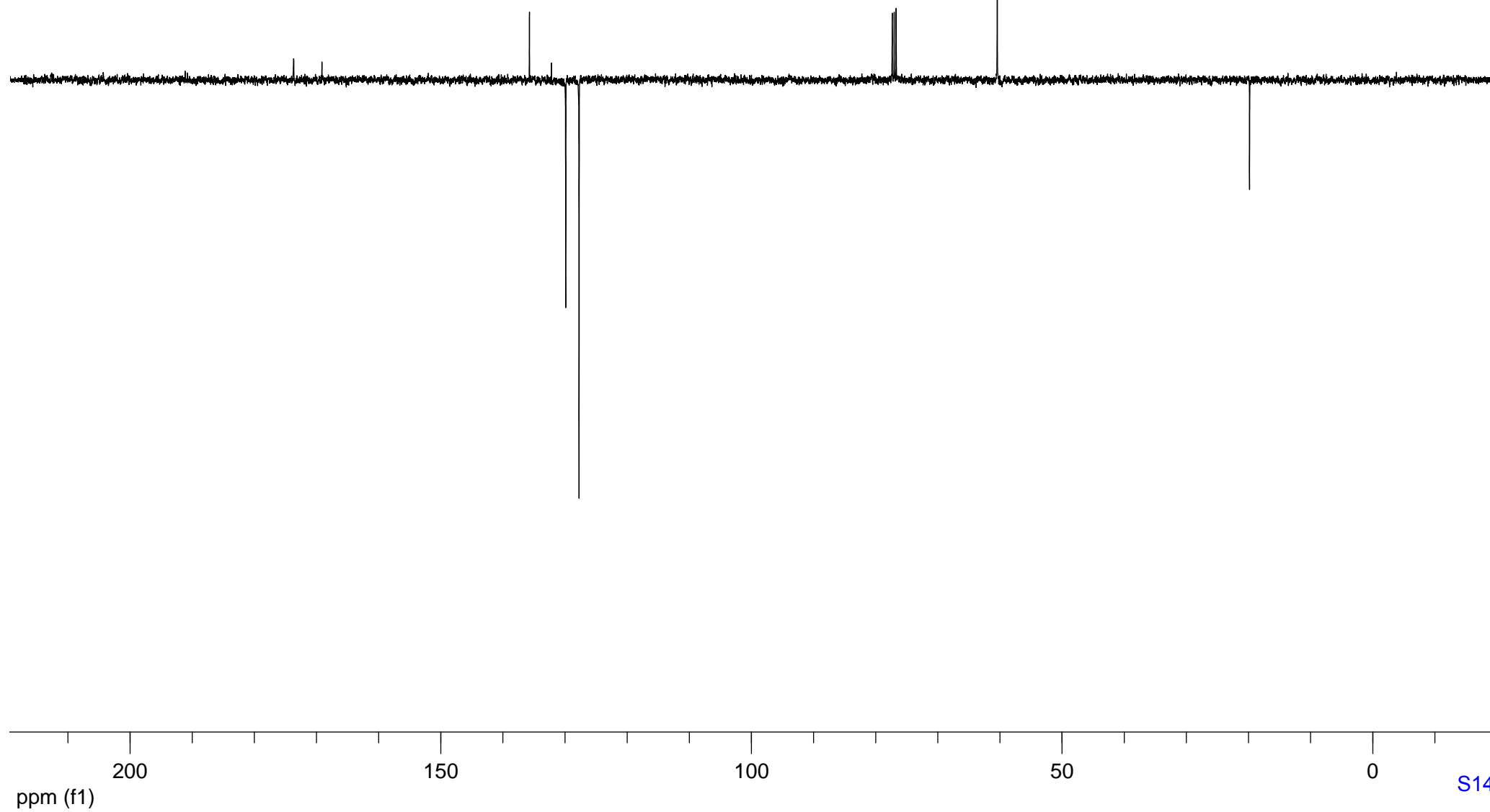
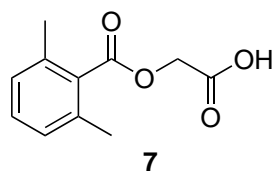
## References

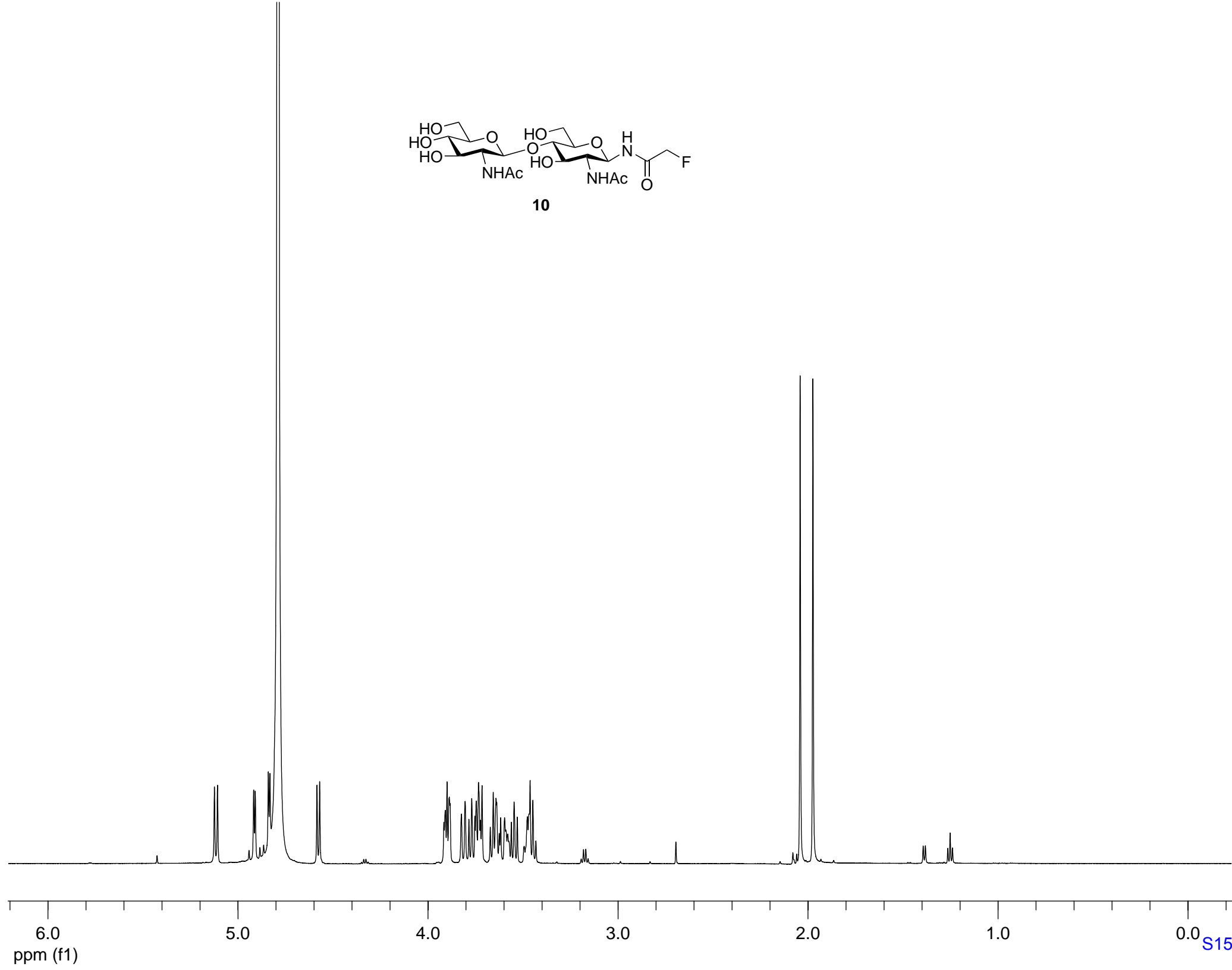
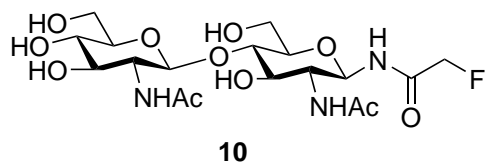
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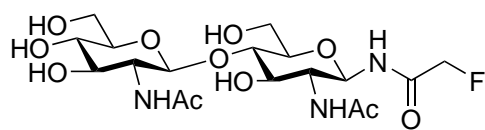




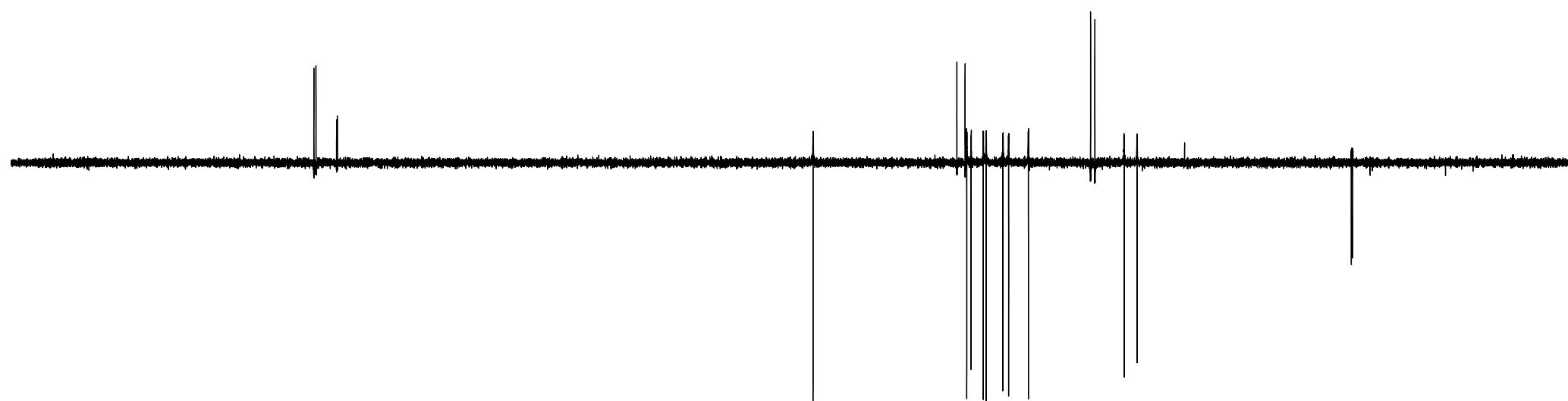








**10**



The figure displays two chromatograms for compound 14. The top panel is the PDA spectrum (uAU vs Time) and the bottom panel is the ESI-MS spectrum (Relative Abundance vs Time). Both panels show retention times for various peaks.

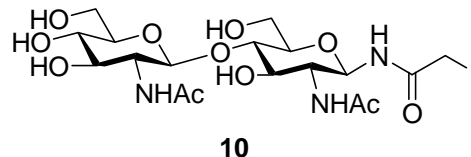
**PDA Spectrum (uAU vs Time):**

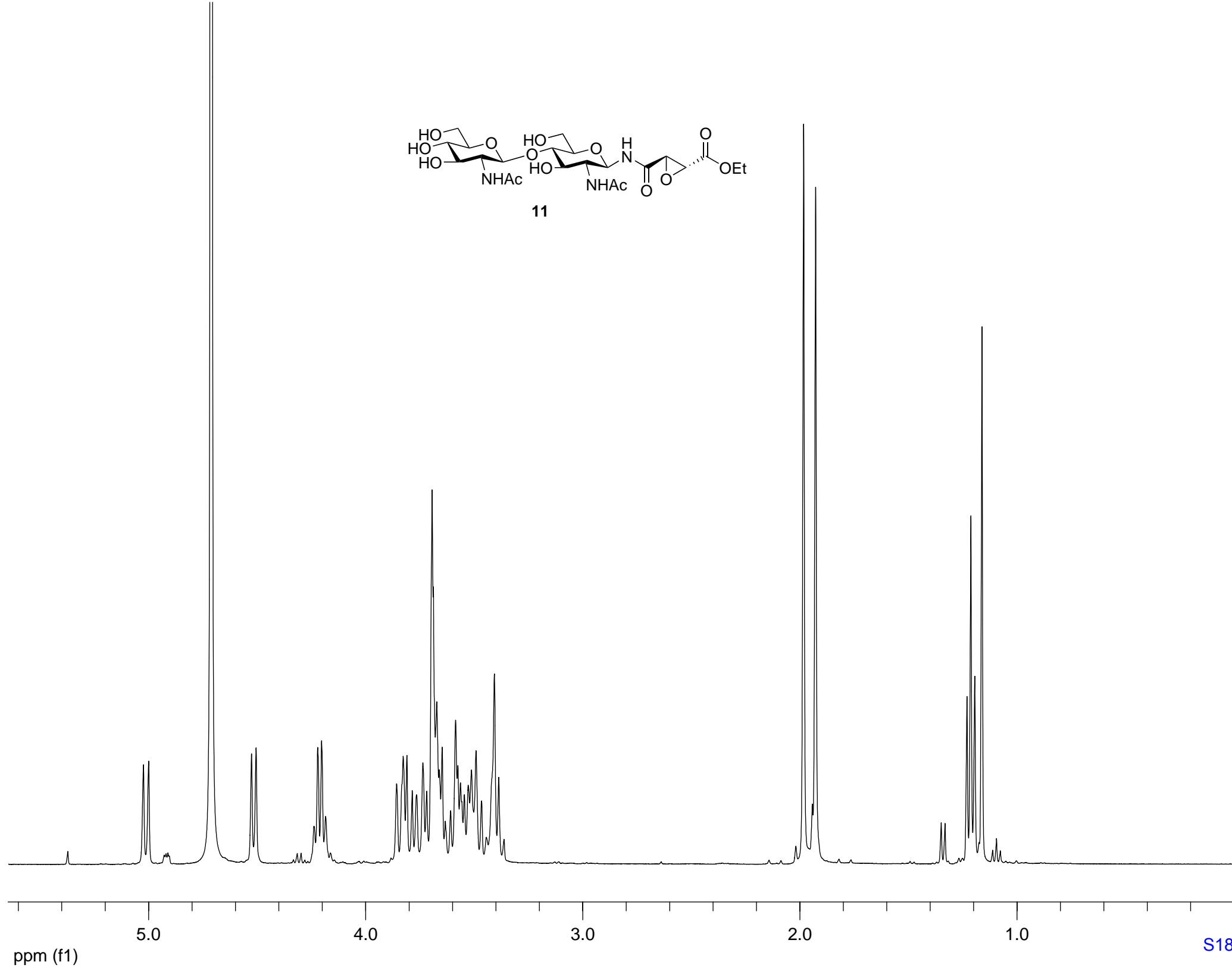
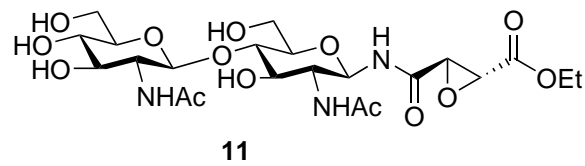
- Retention times: 0.61, 0.69, 1.28, 1.45, 2.78, 3.09, 3.65, 4.98, 6.03, 6.58, 7.39, 7.77, 8.73, 9.20, 10.03, 10.37, 11.86, 12.30, 14.24.
- Peak at 14.24: NL: 7.37E5, Spectrum Maximum, nm=200.0-600.0, PDA mdw656.

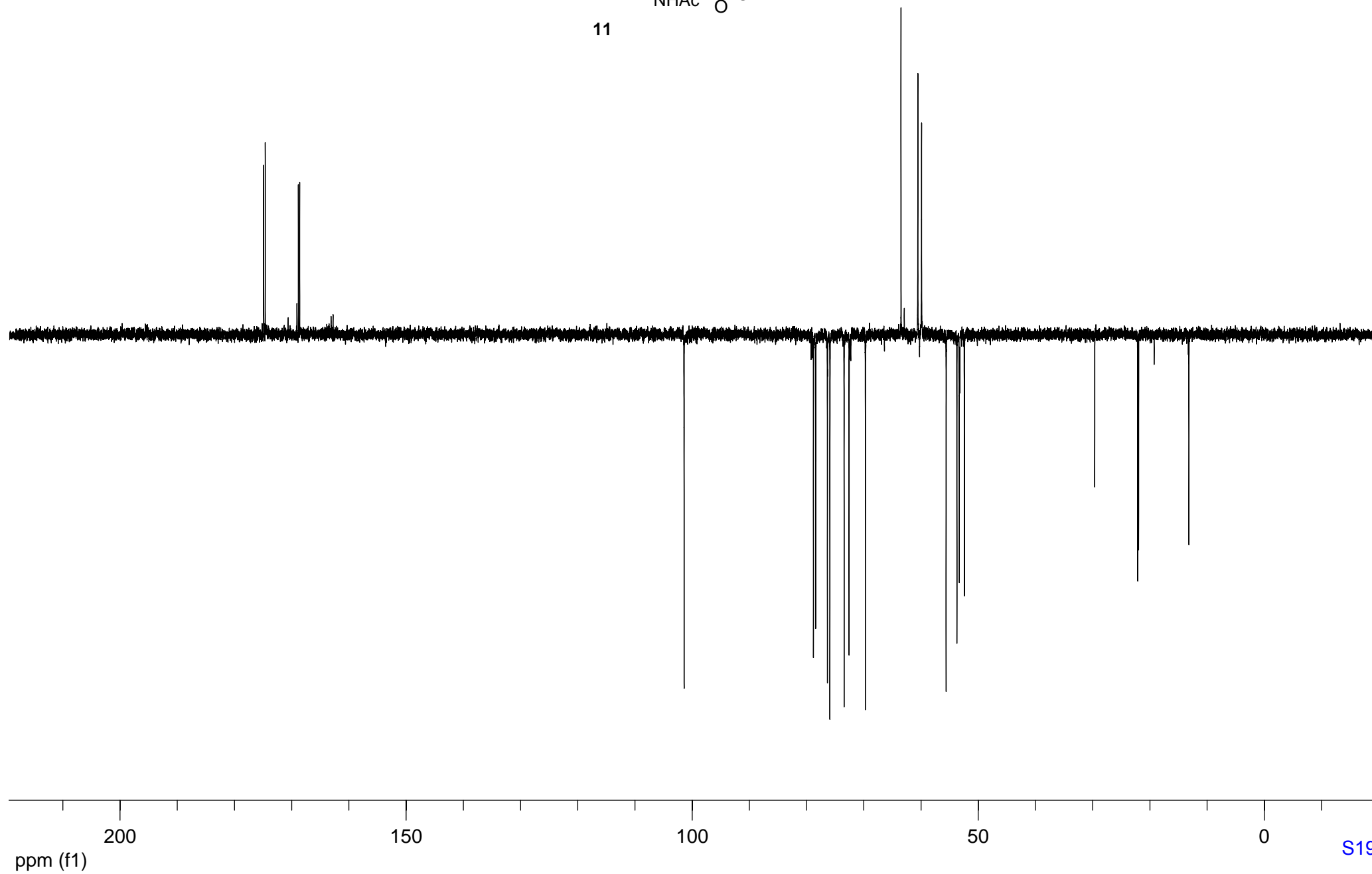
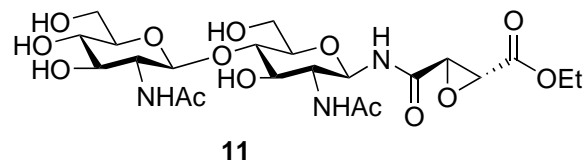
**ESI-MS Spectrum (Relative Abundance vs Time):**

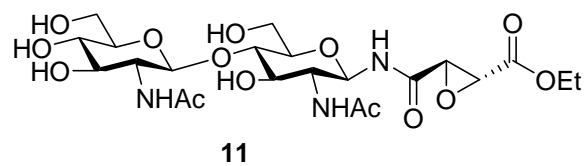
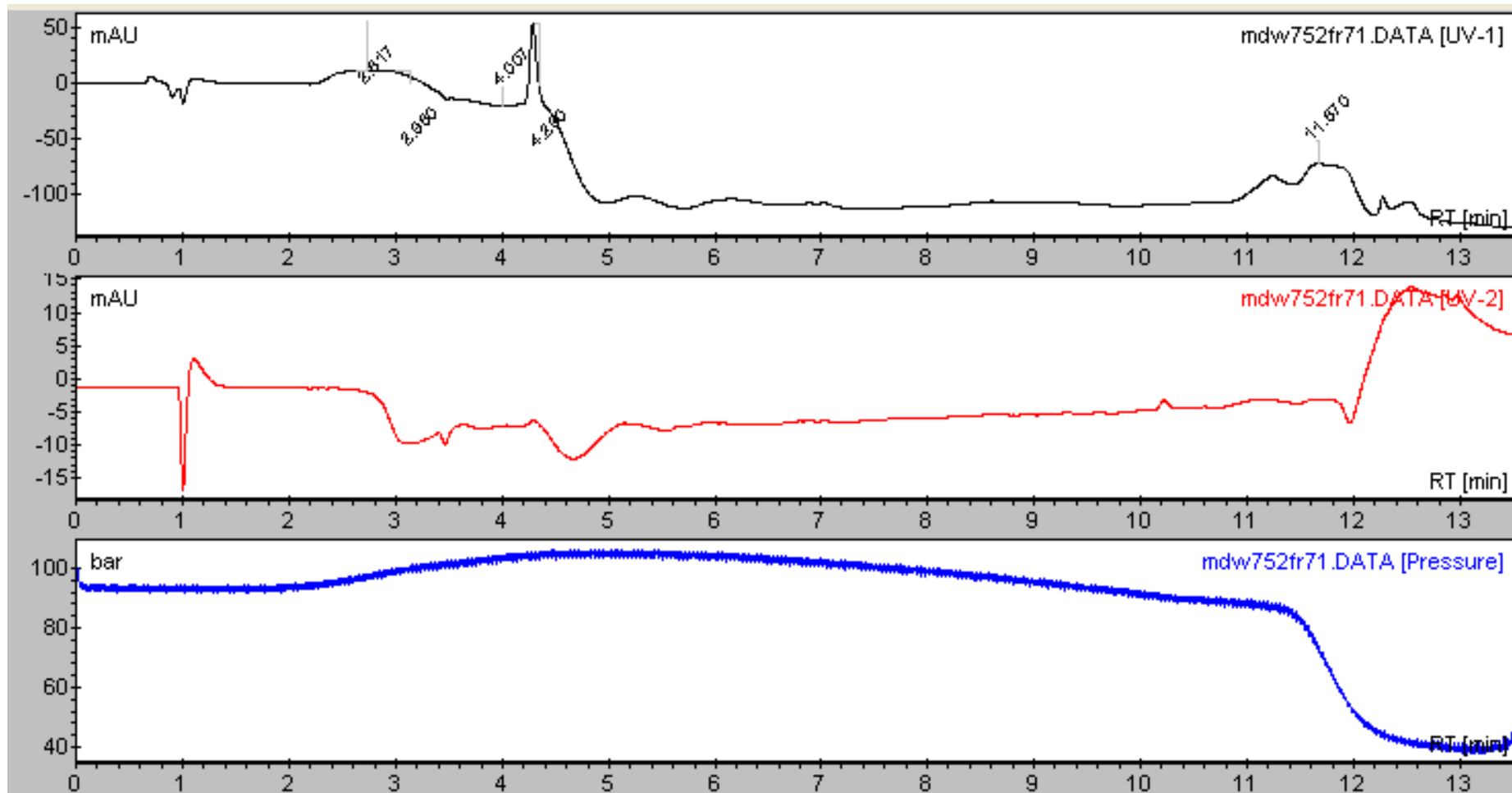
- Retention times: 0.02, 0.71, 0.90, 1.31, 1.44, 1.64, 2.03, 2.90, 3.06, 3.61, 4.15, 4.72, 5.33, 6.54, 7.29, 7.76, 8.39, 9.24, 9.93, 10.62, 11.25, 11.94, 12.40, 12.53, 12.99, 13.86.
- Peak at 12.40: NL: 1.63E6, Base Peak F: + p, ESI Full ms [160.00-2000.00], MS mdw656.

mdw656 #68 RT: 1.33 AV: 1 NL: 1.19E6  
F: + p ESI Full ms [ 160.00-2000.00]

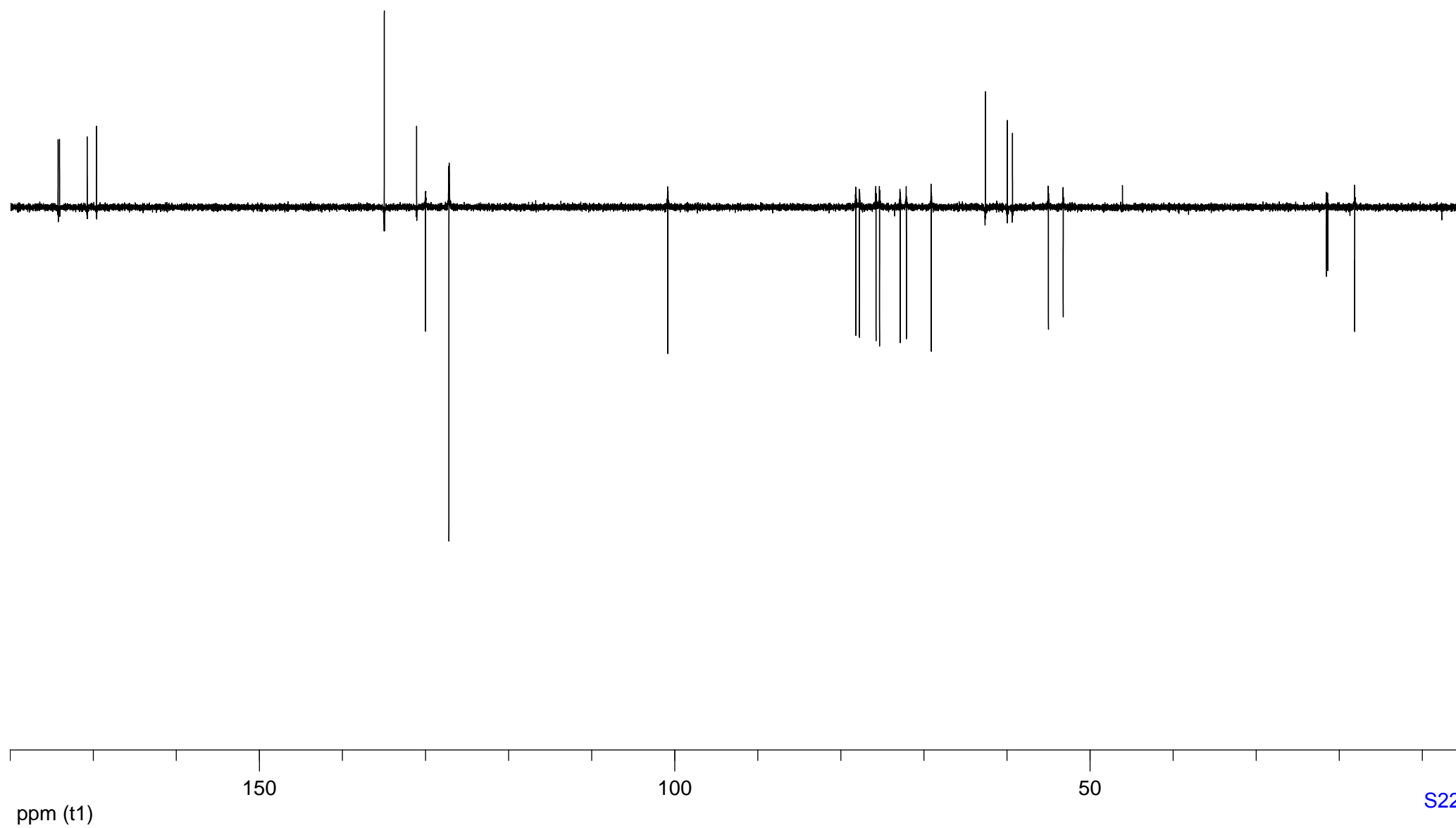
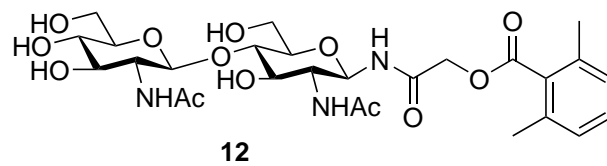


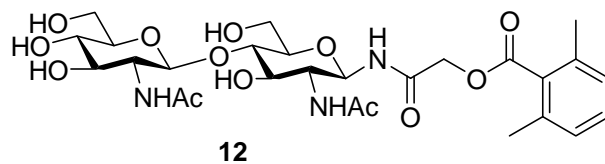
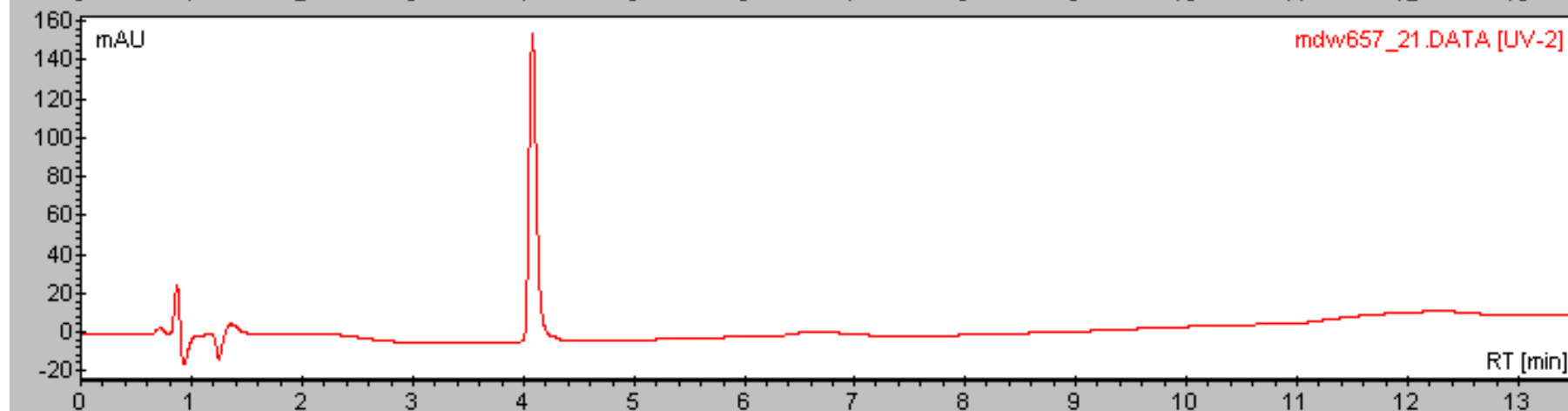
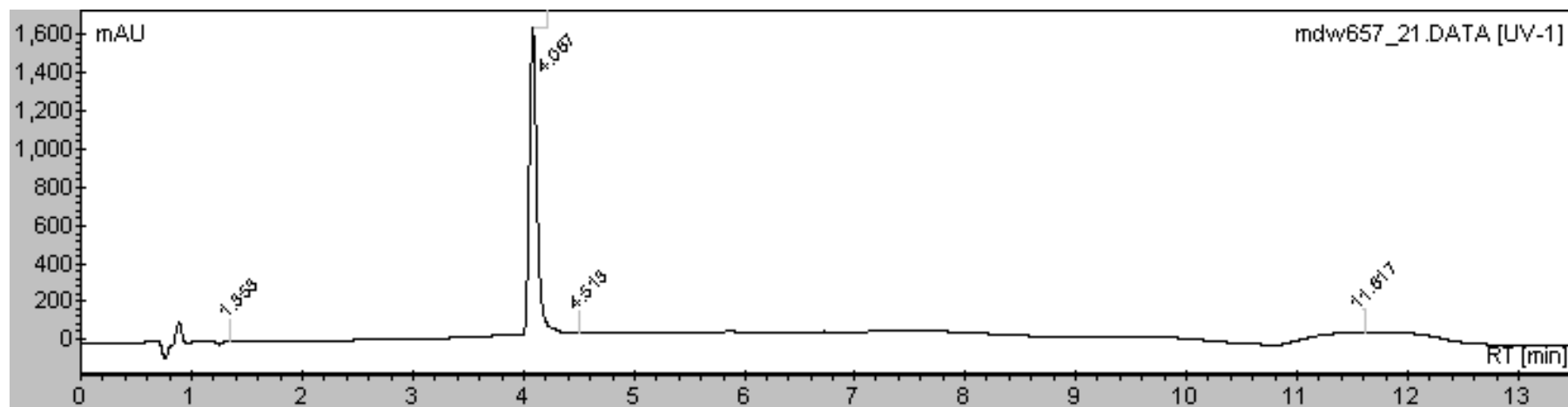




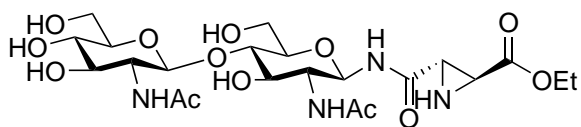




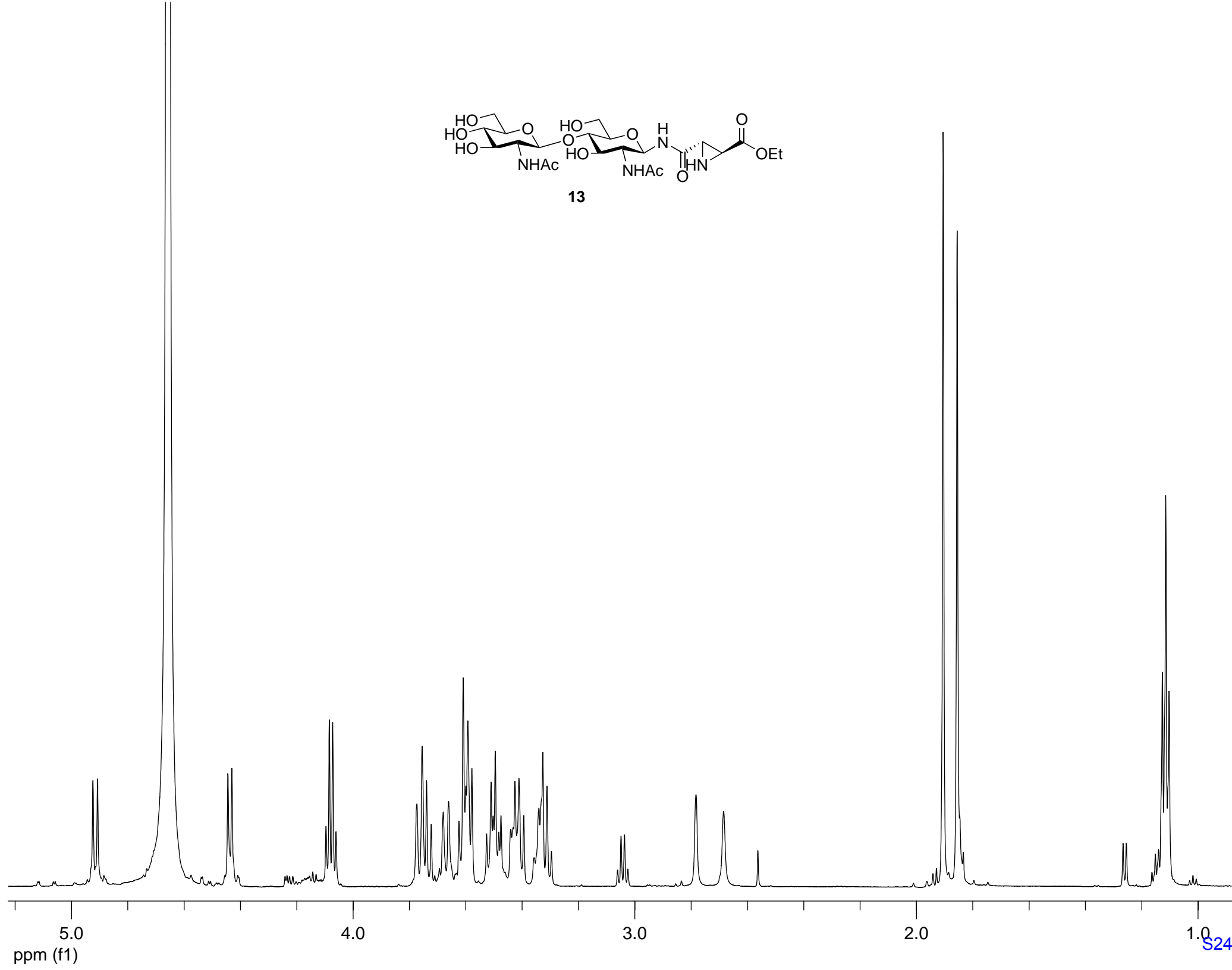


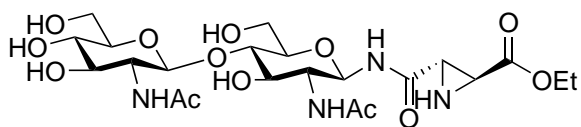




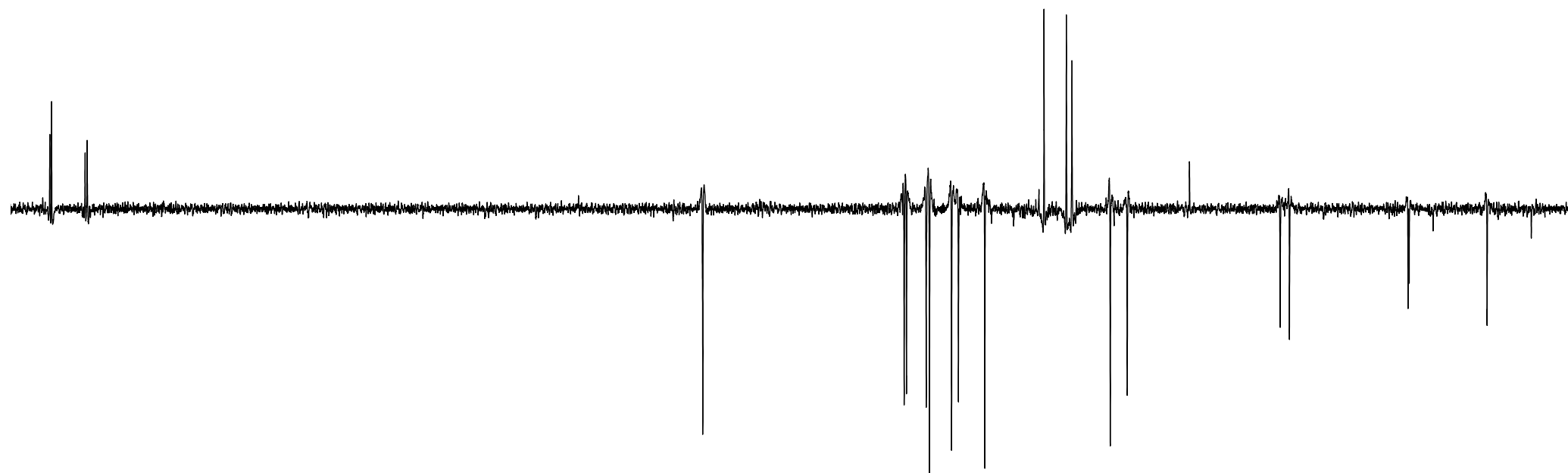


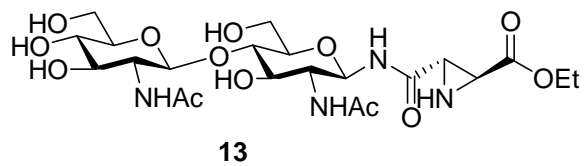
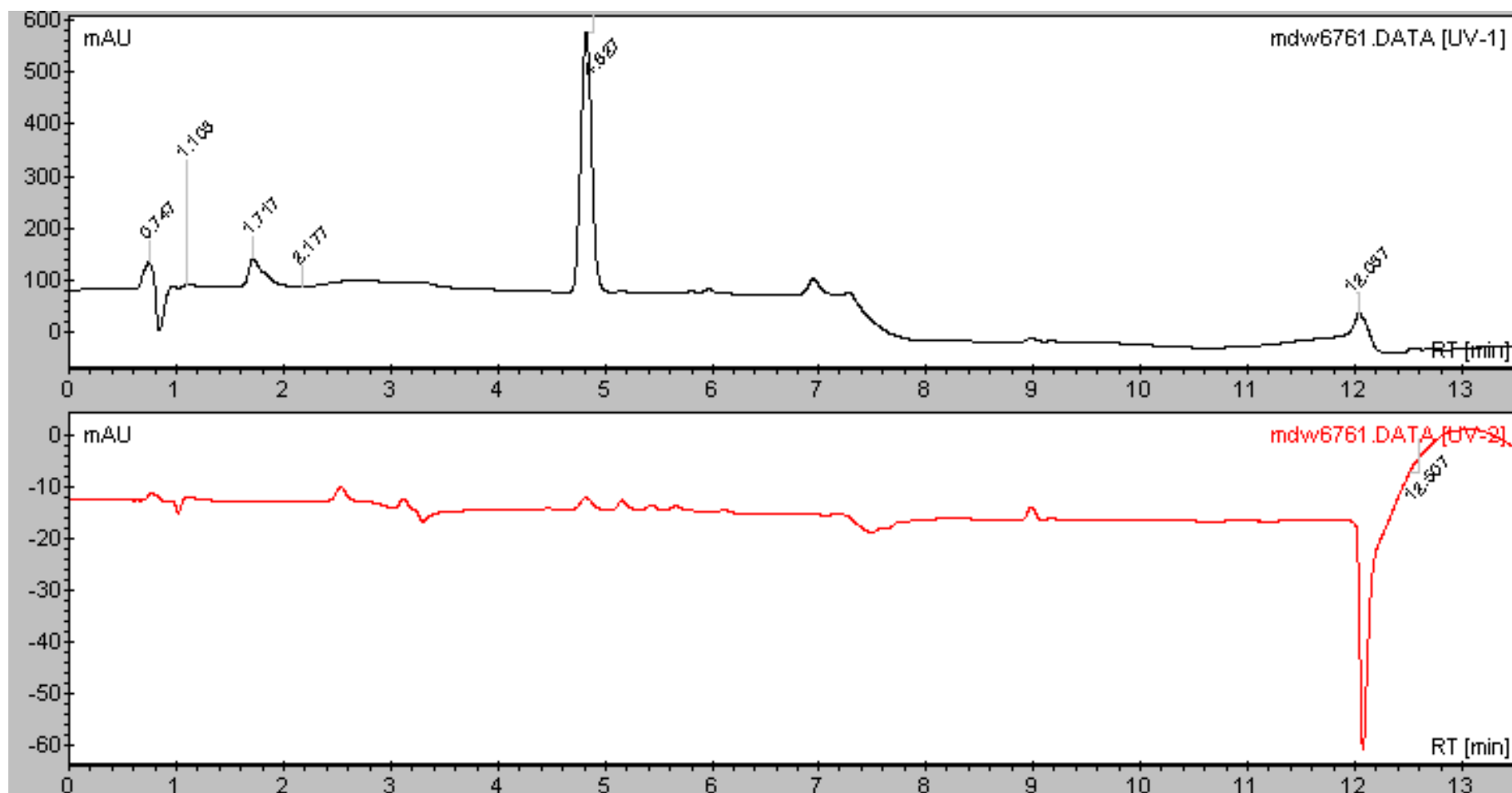
**13**

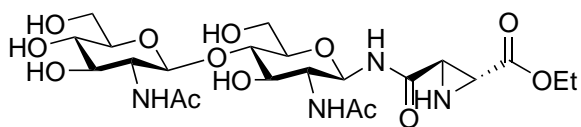




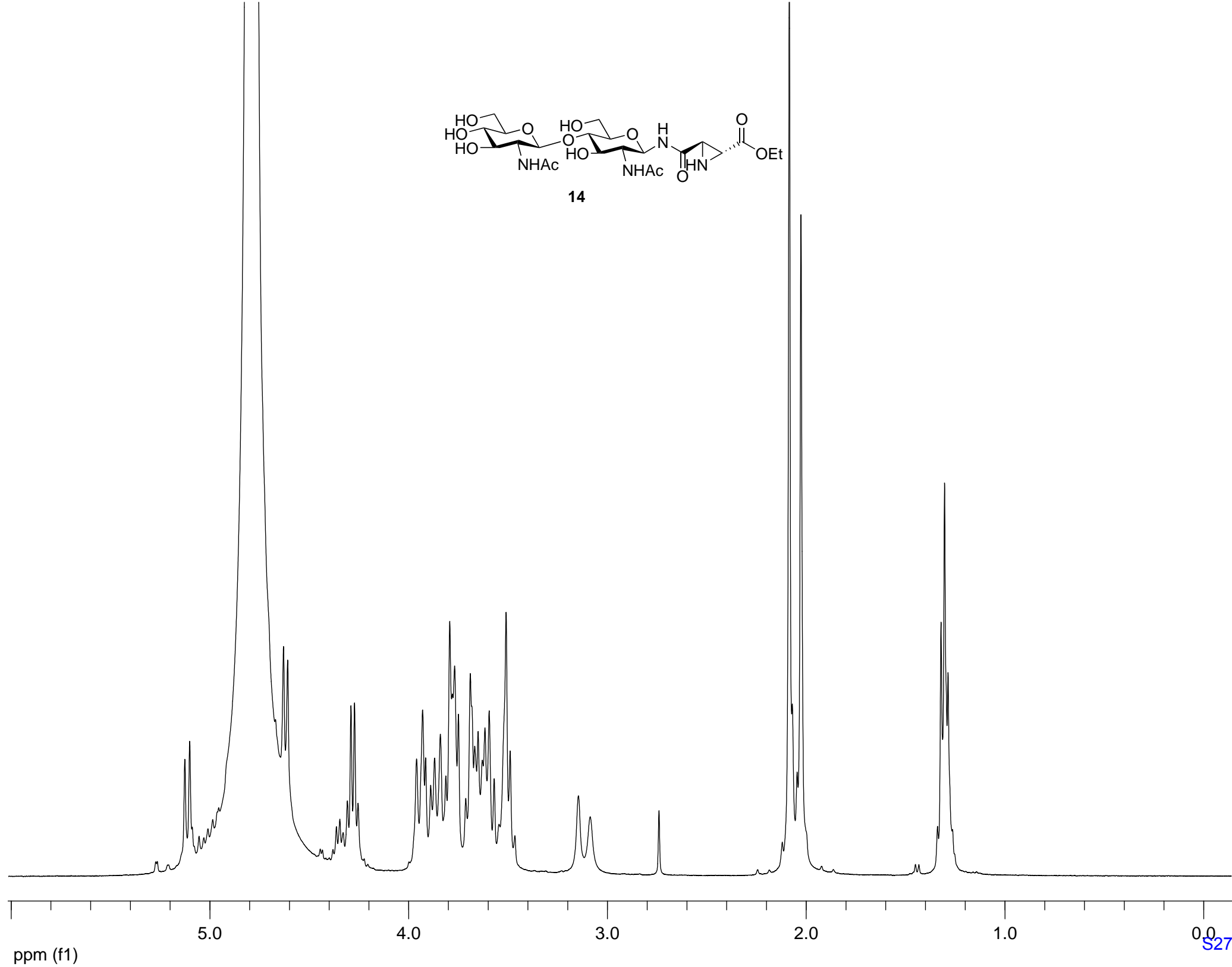
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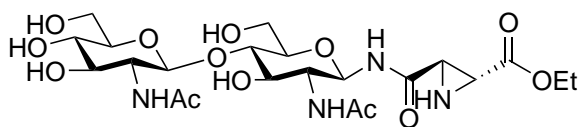




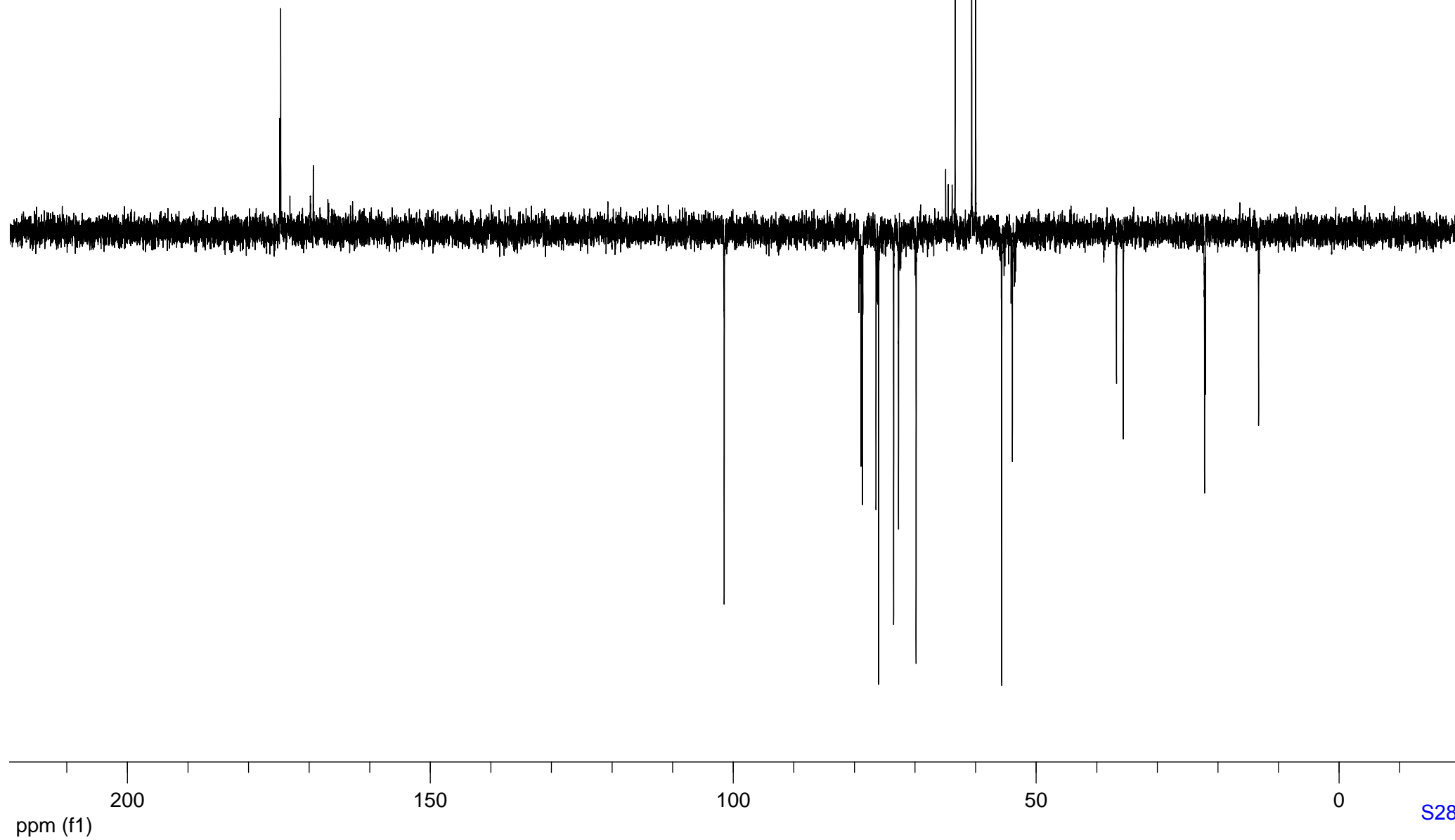


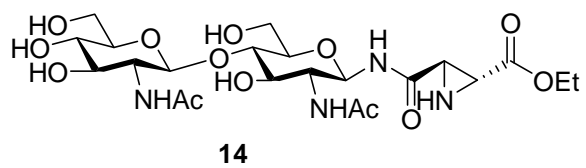
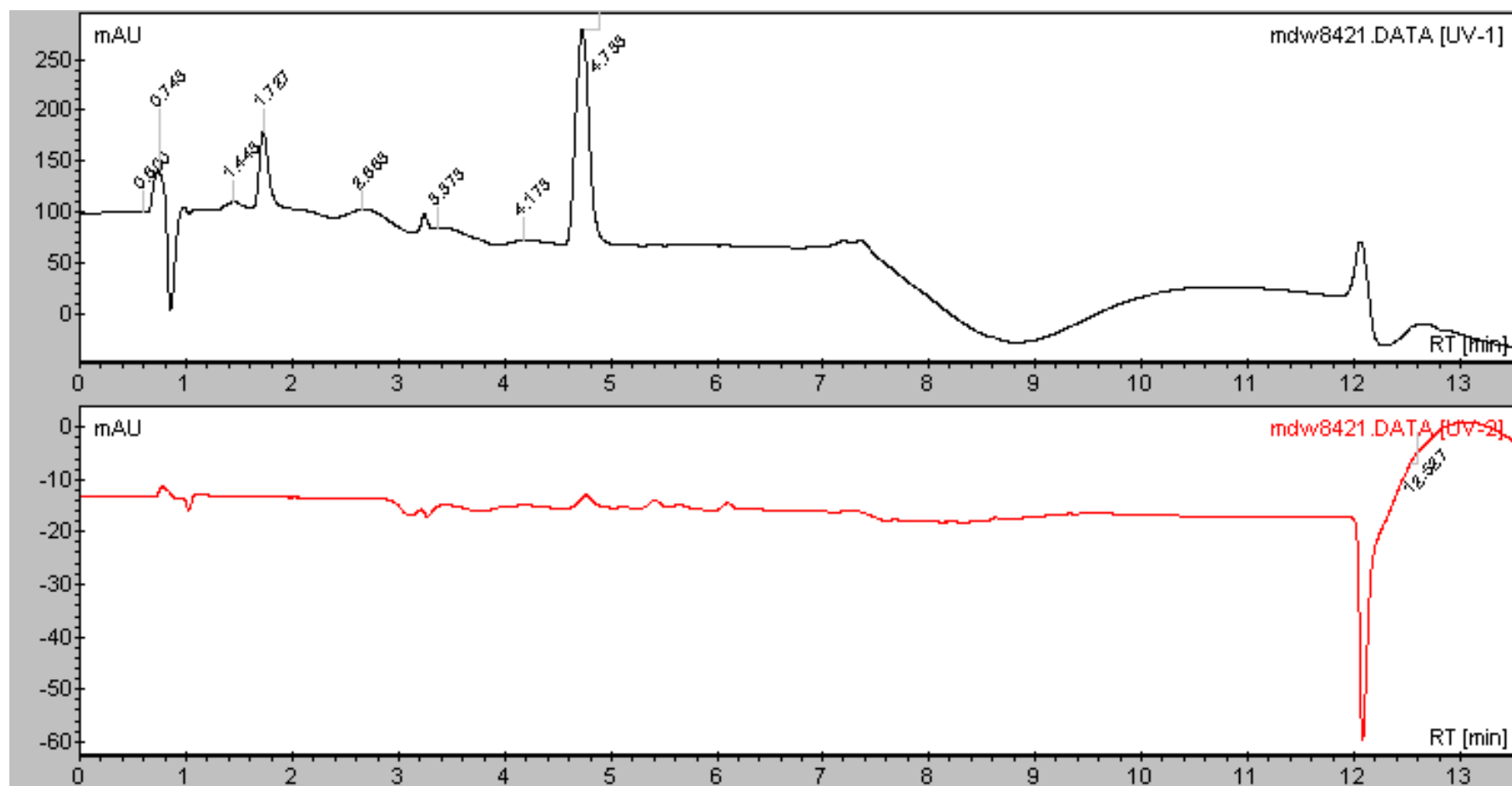
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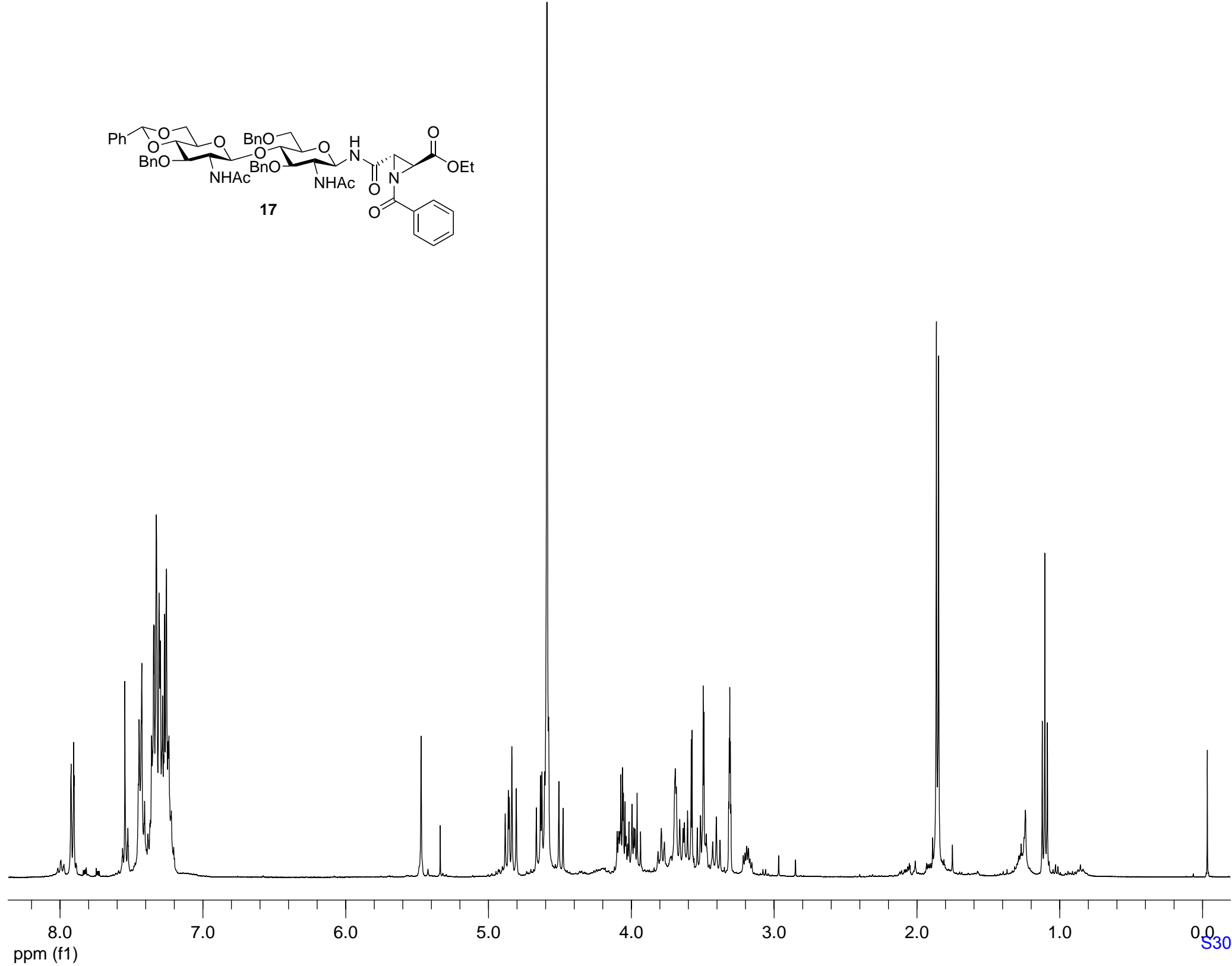
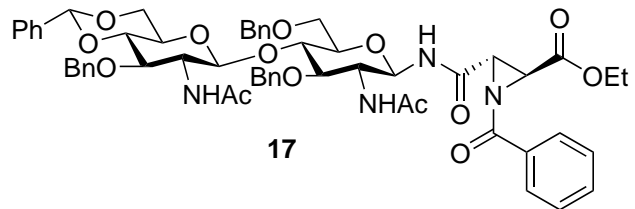


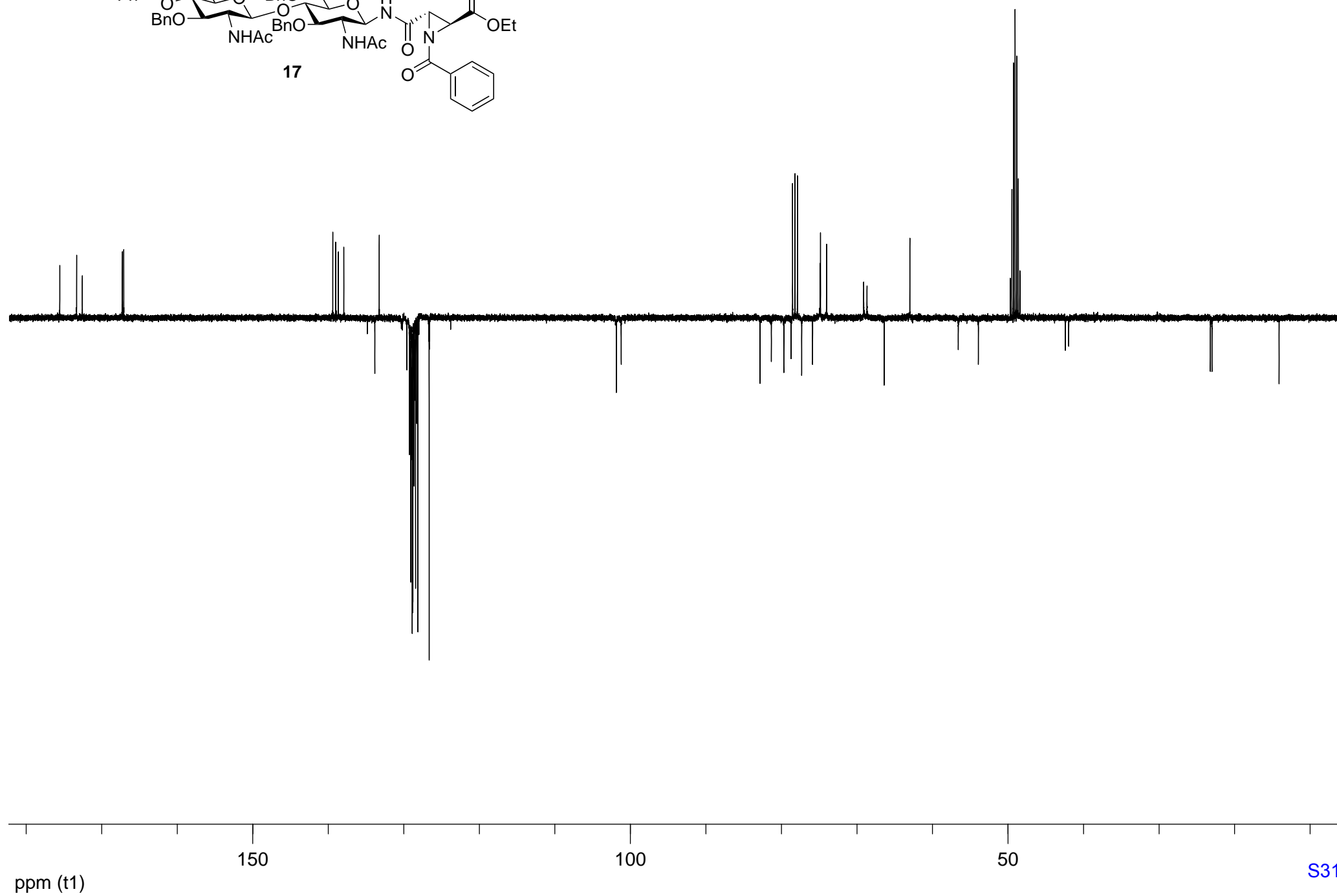
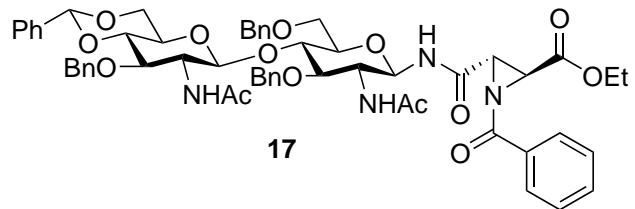


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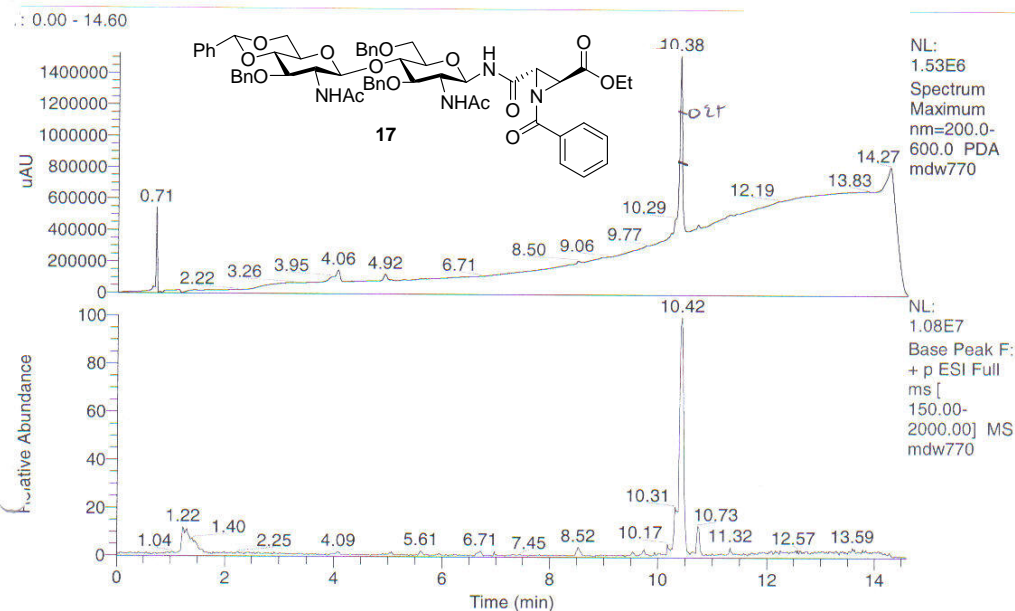






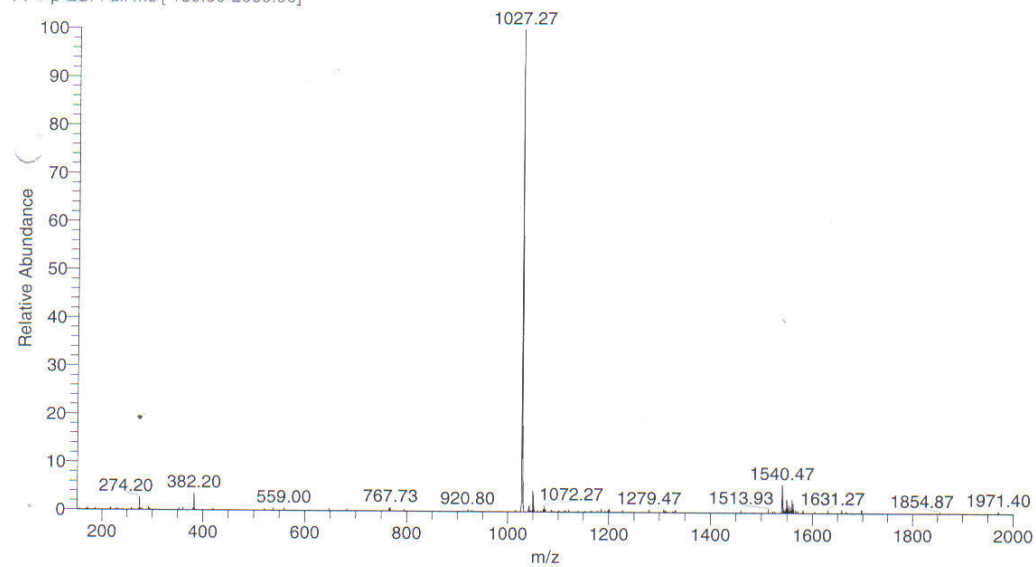


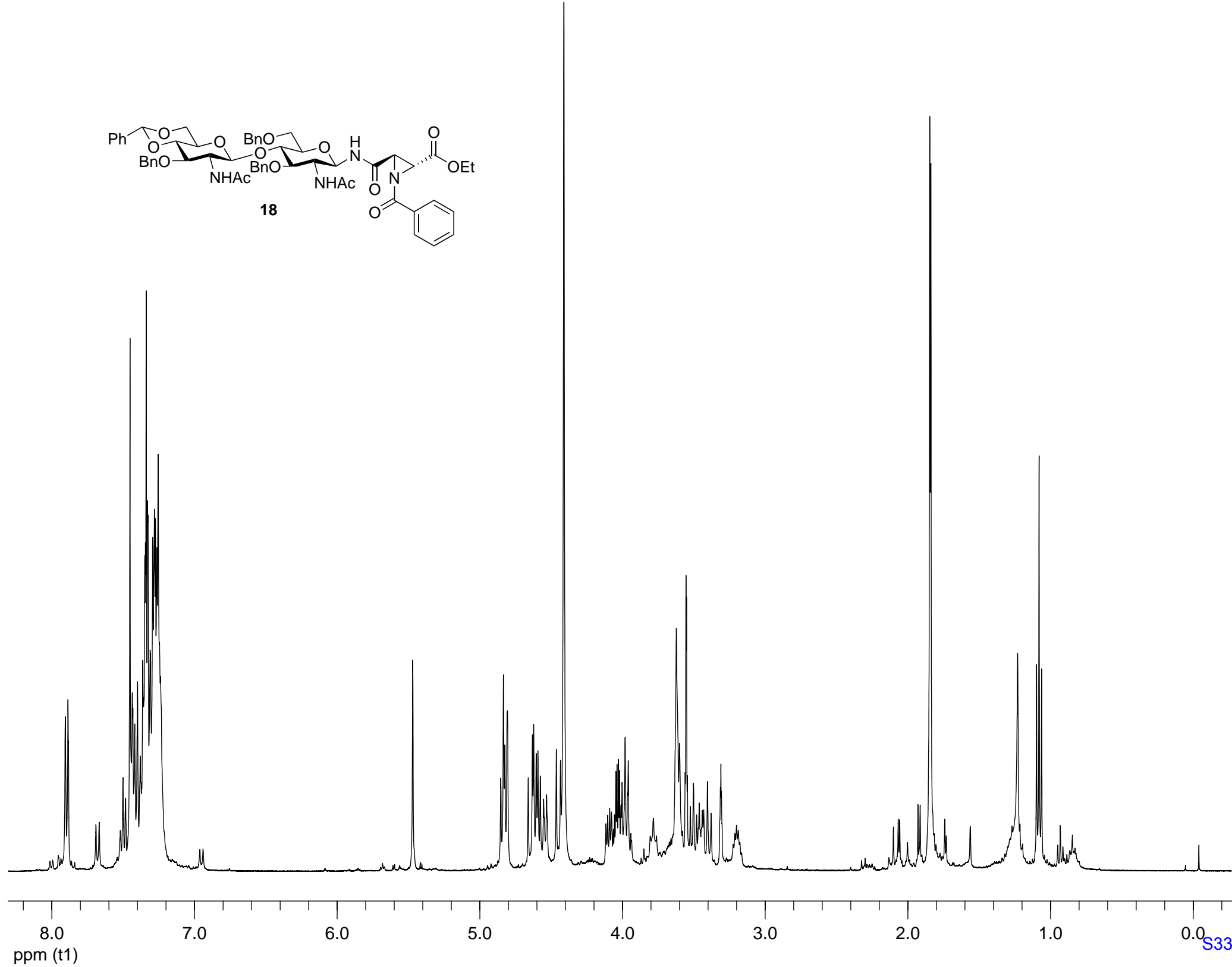
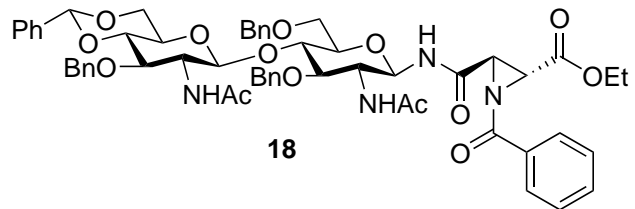


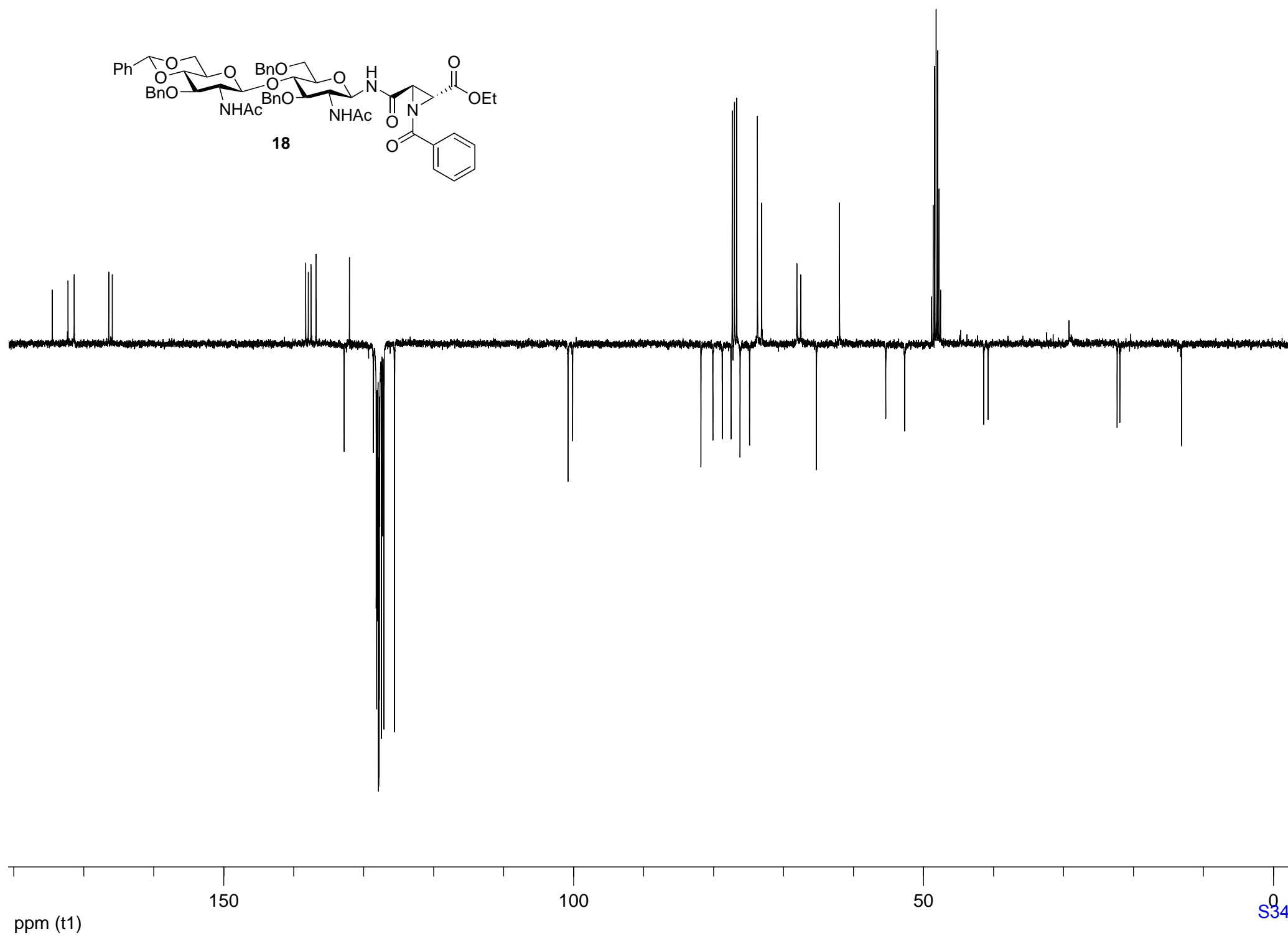
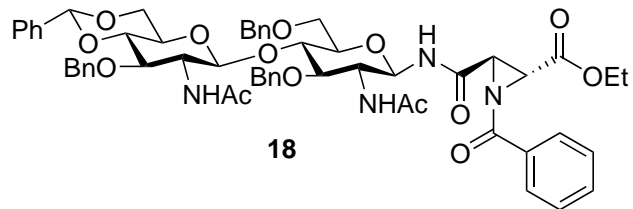


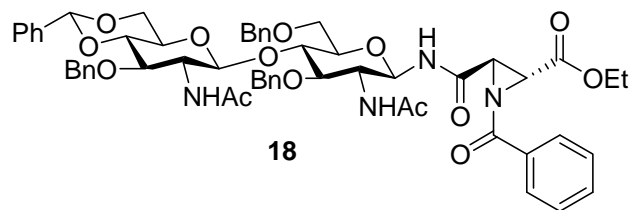
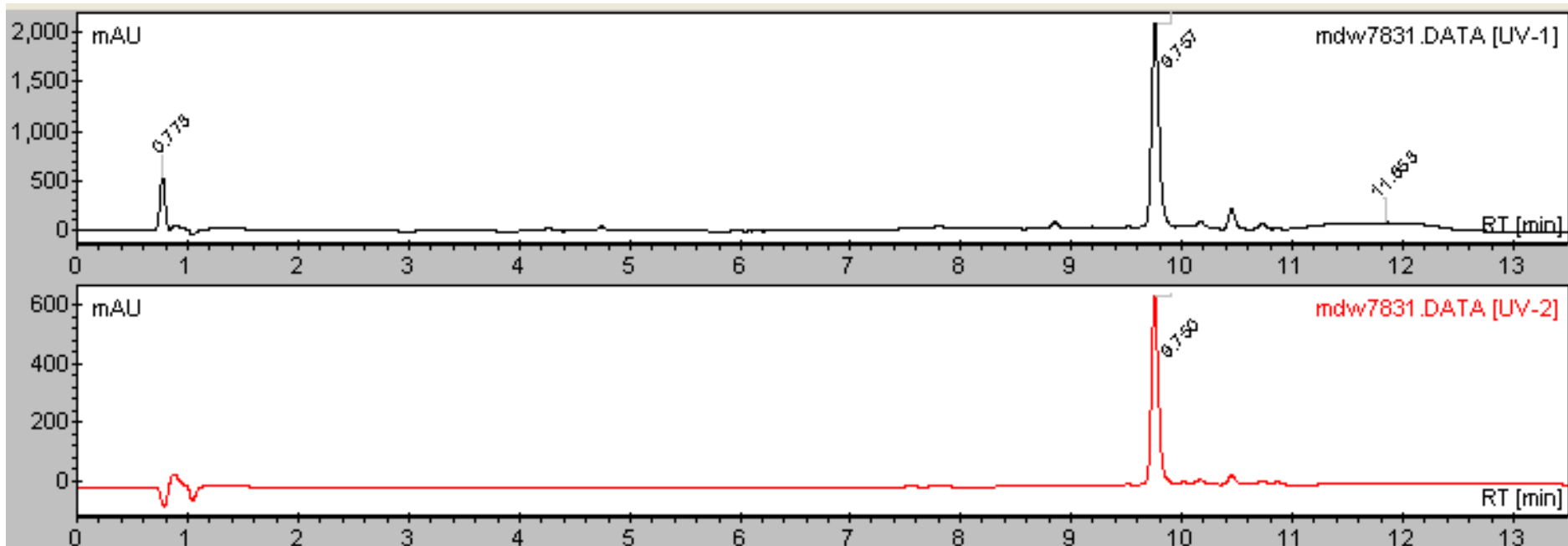
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Laboratory:

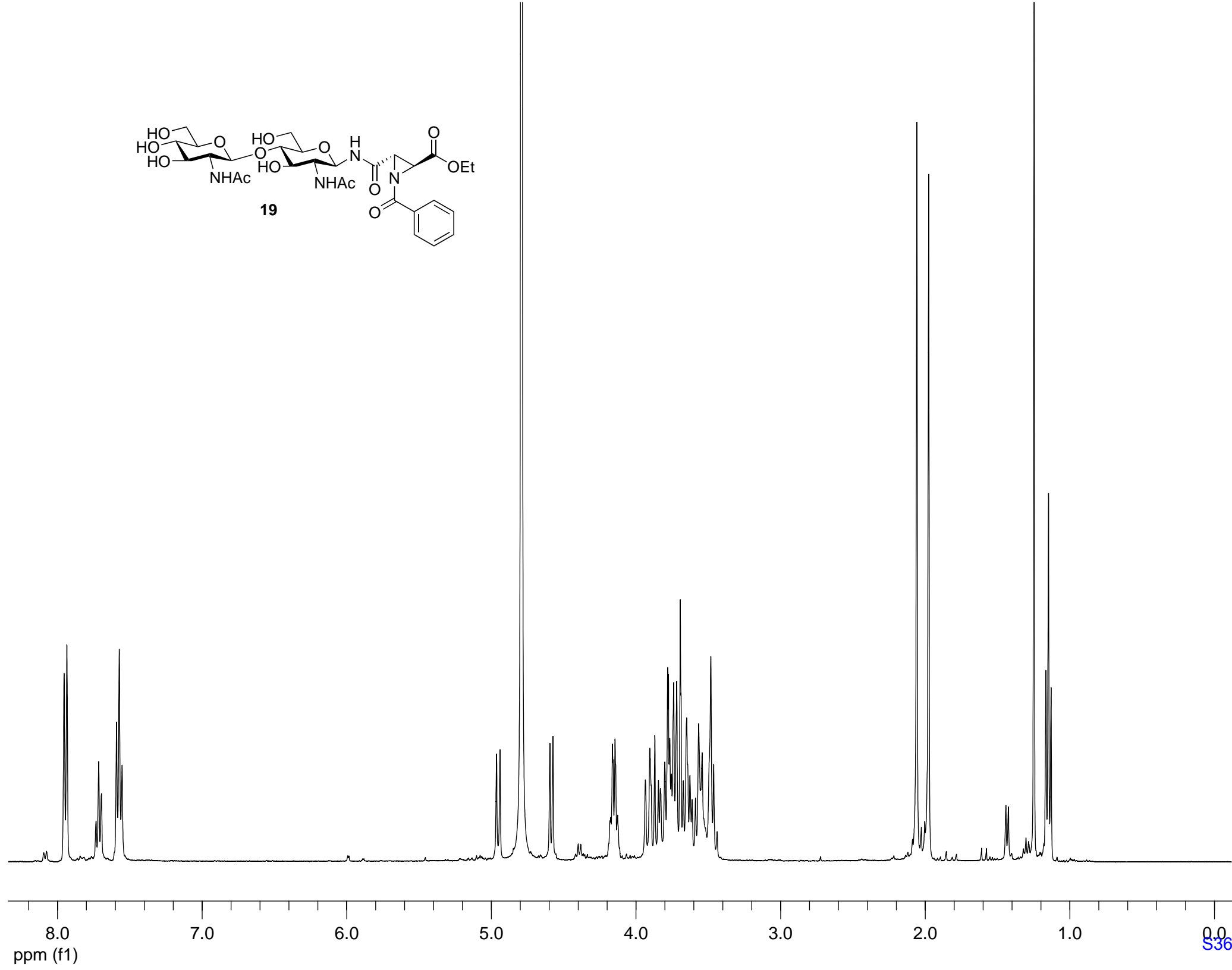
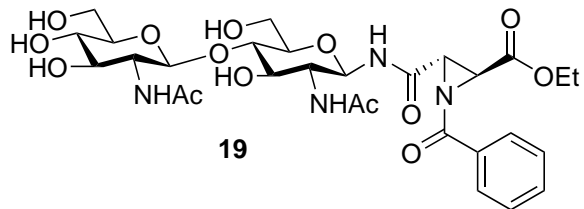
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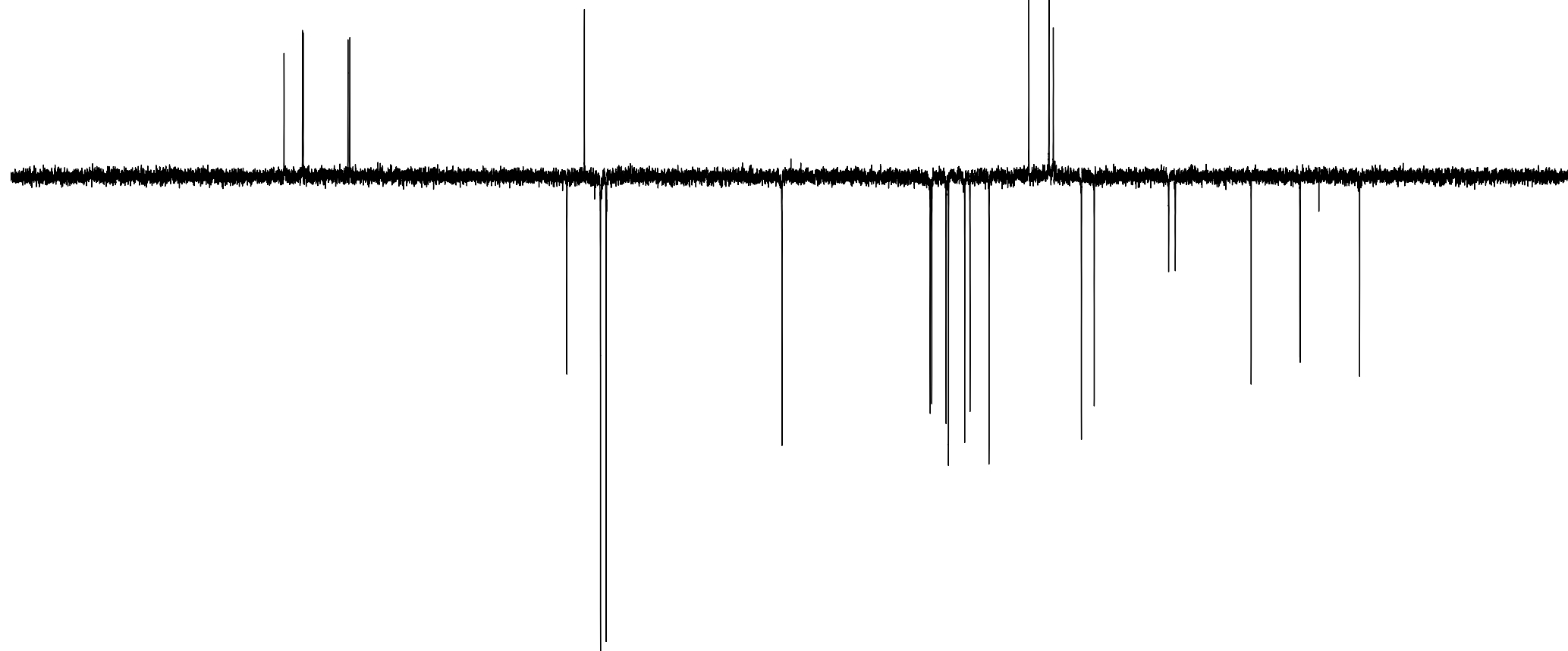
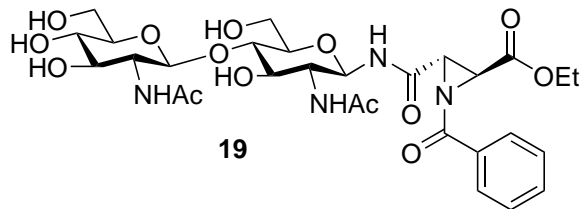


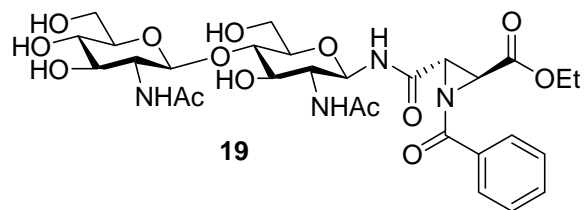
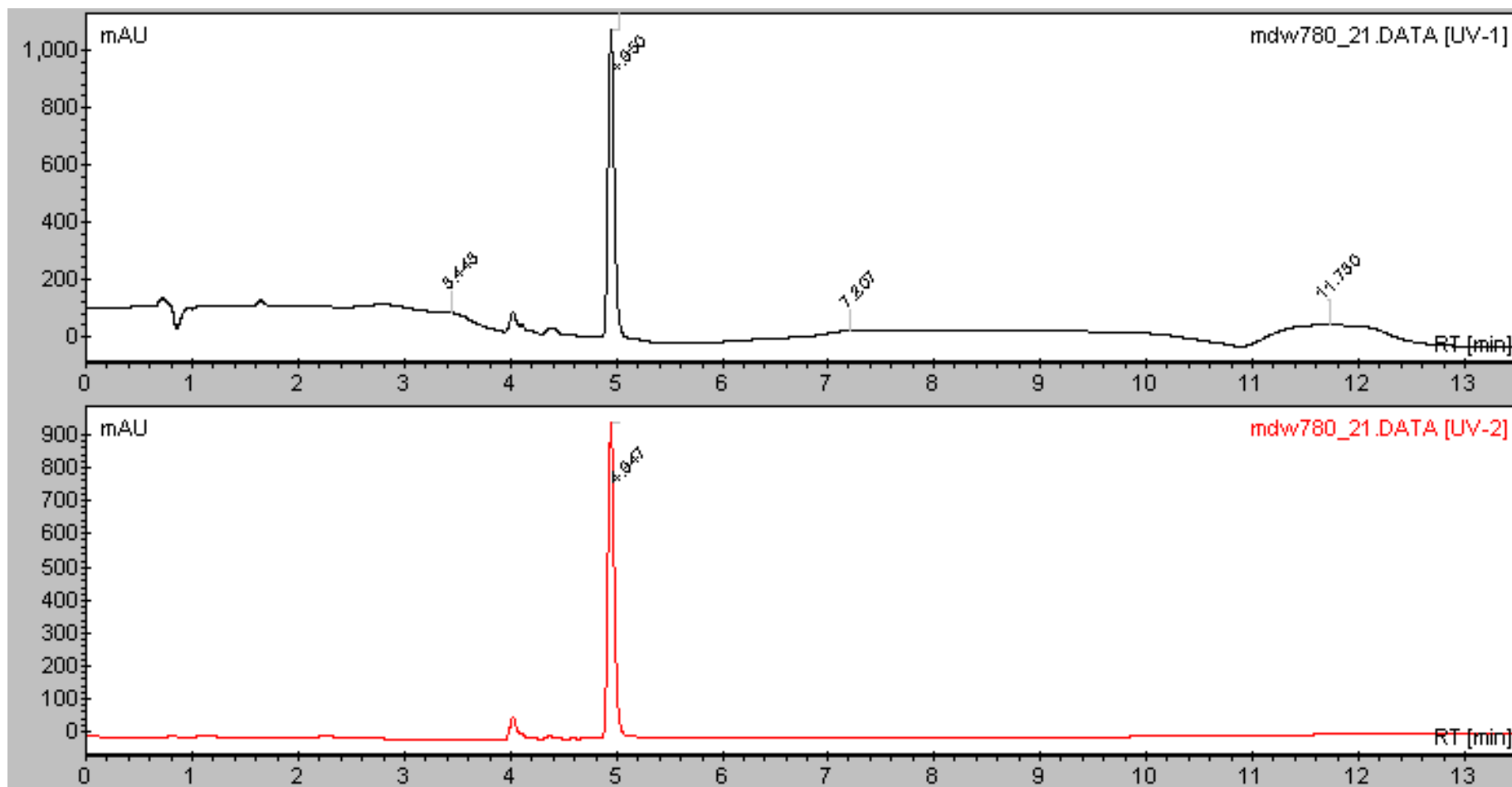


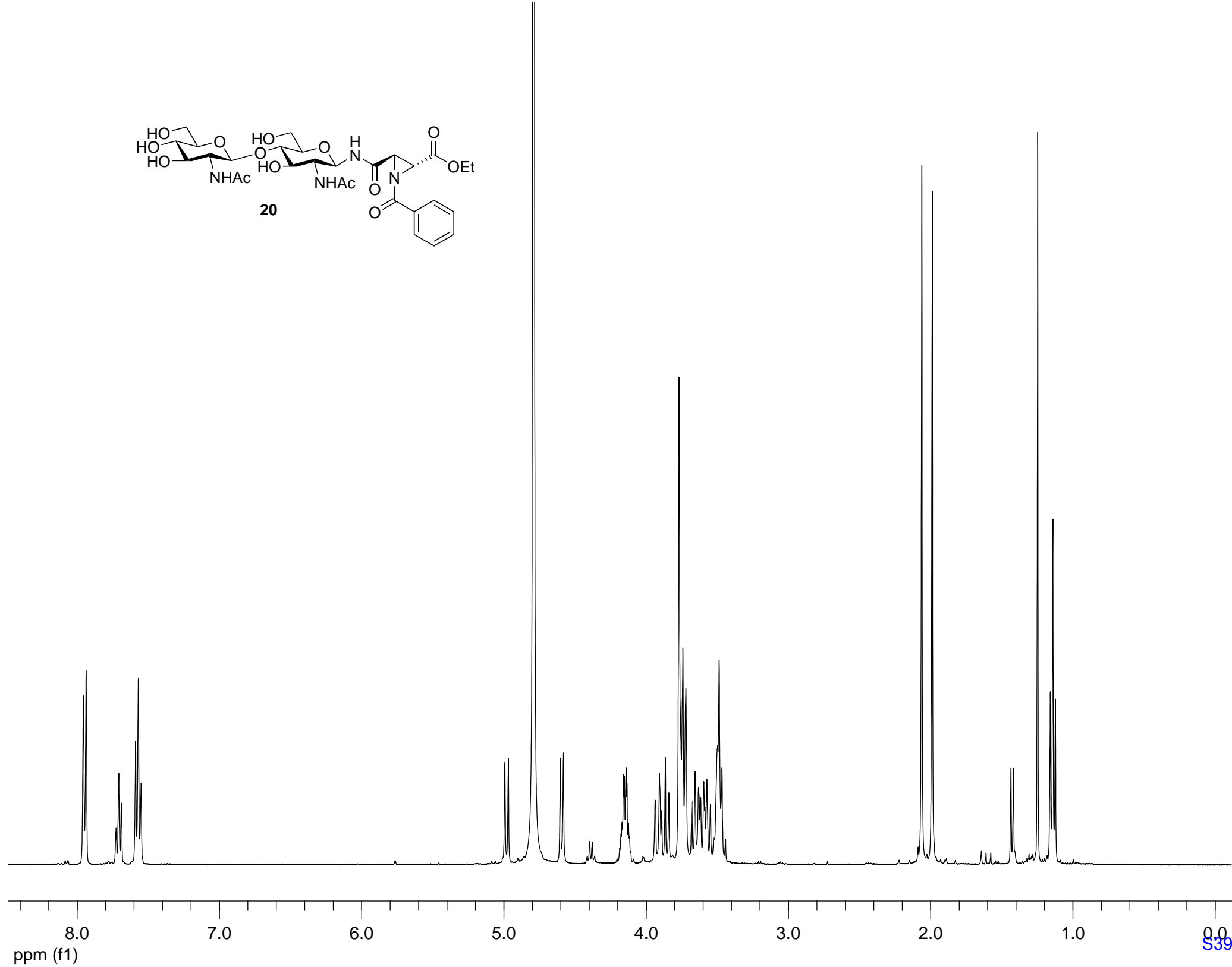
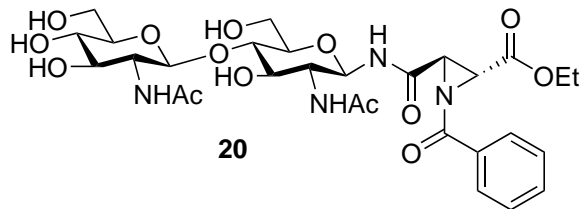




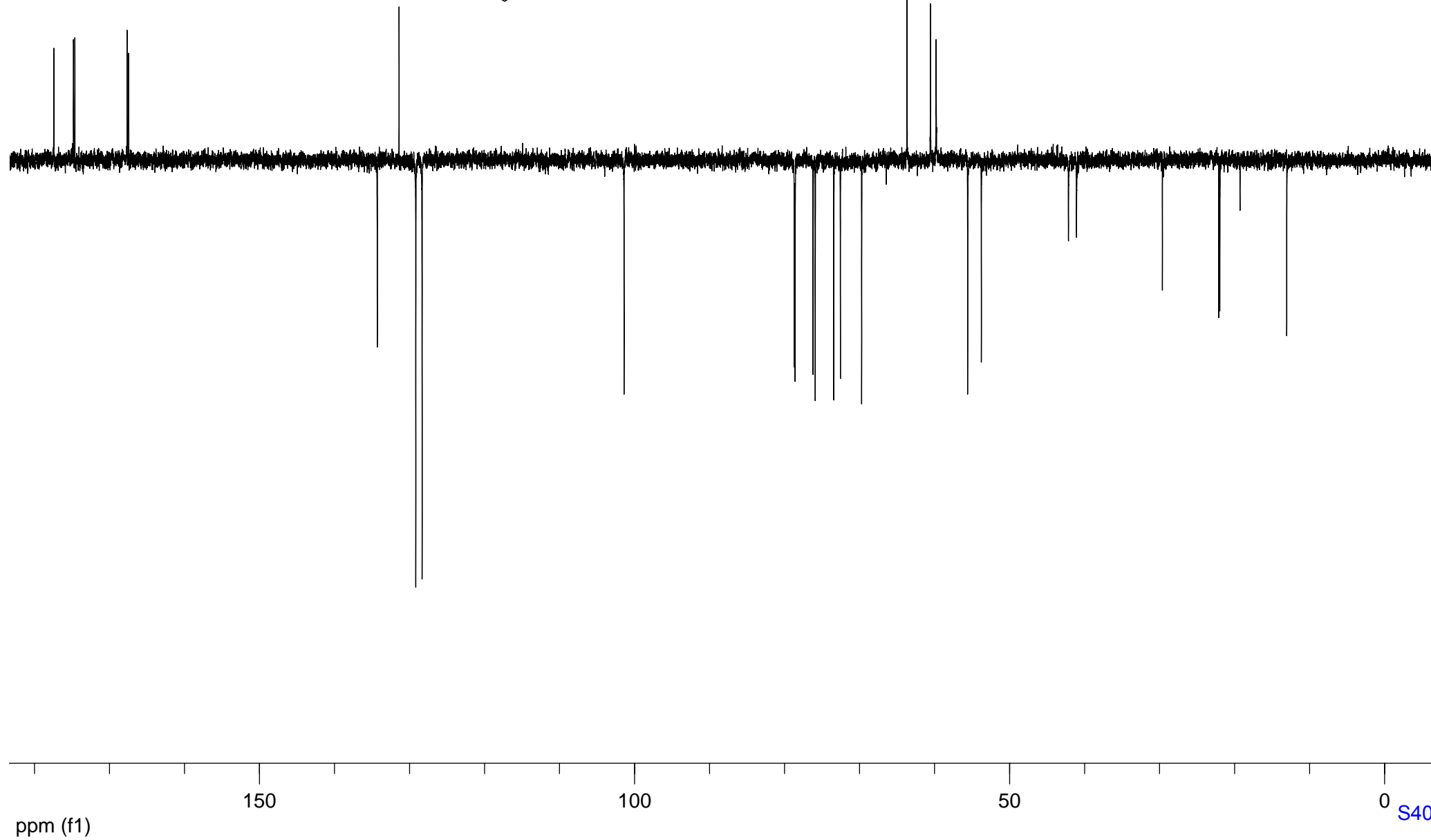
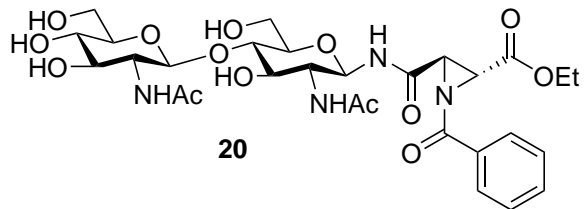


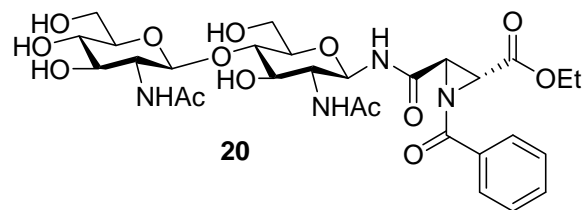
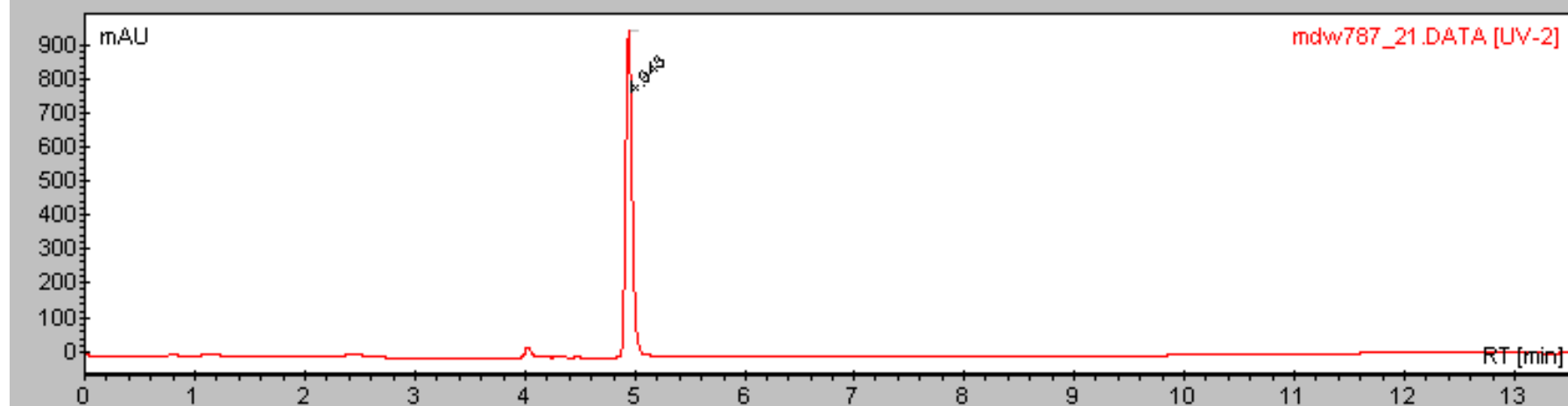
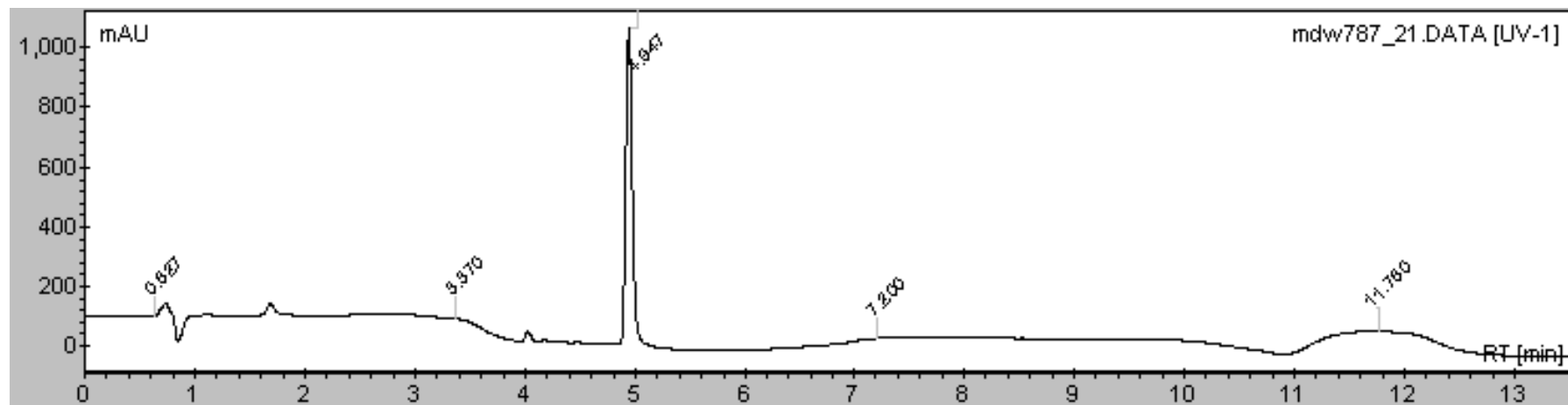




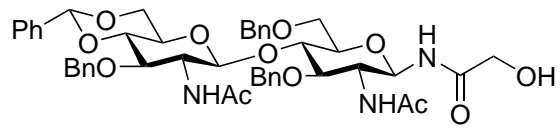




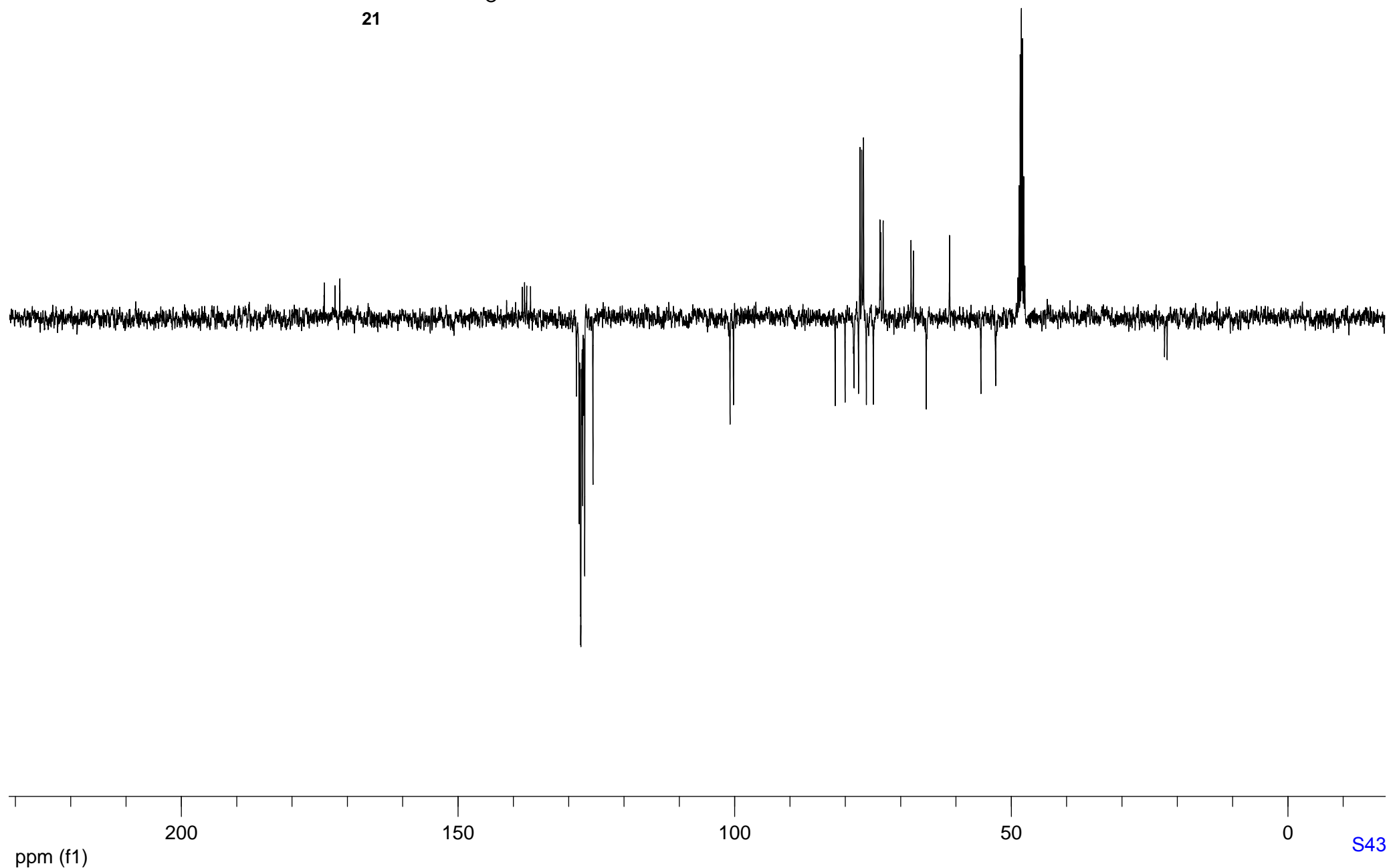


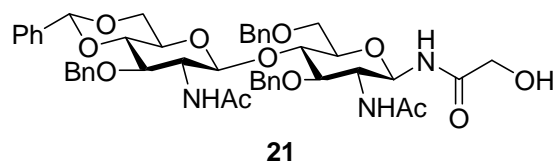
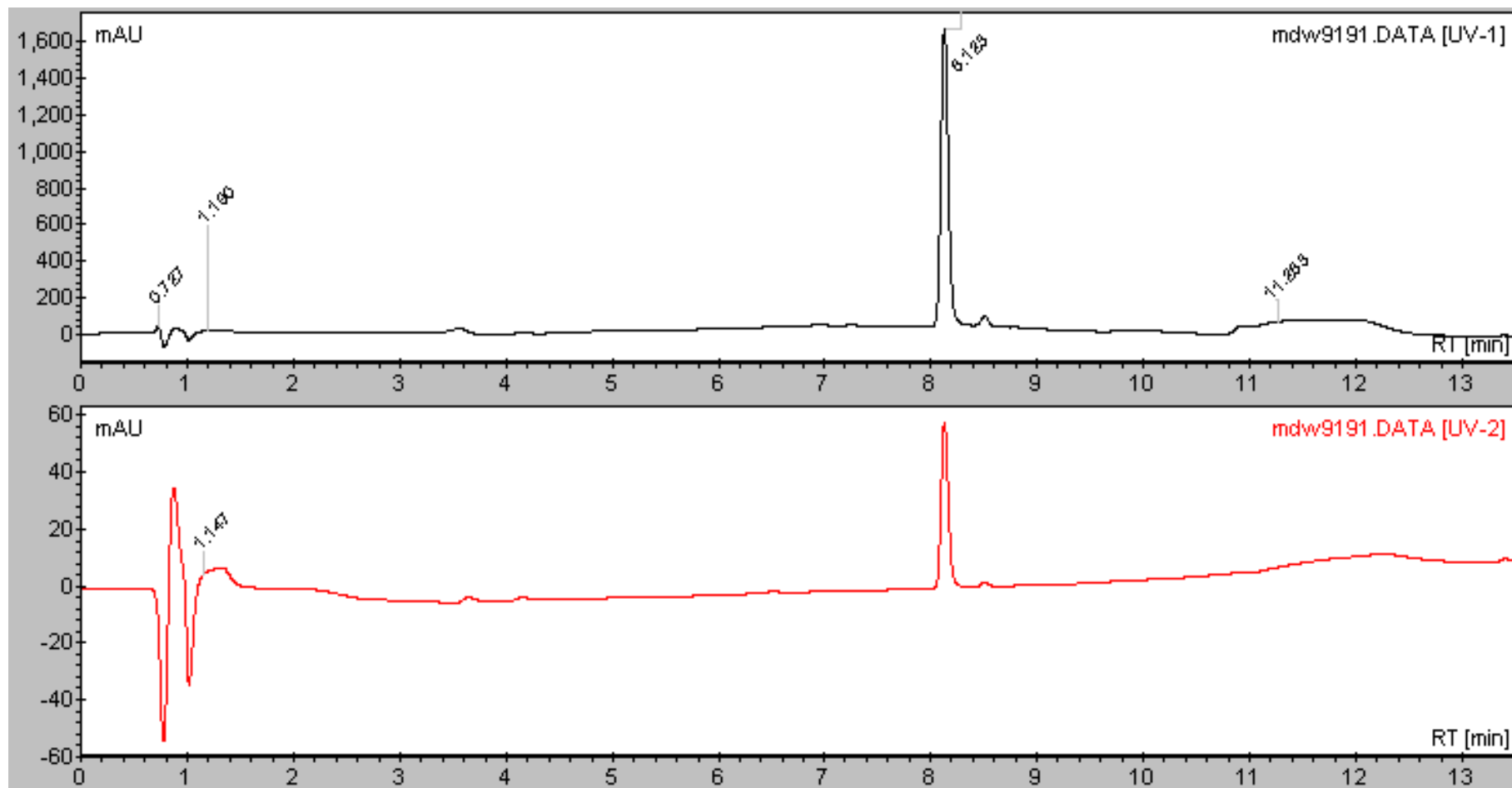


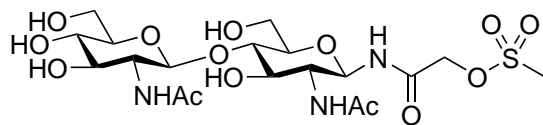




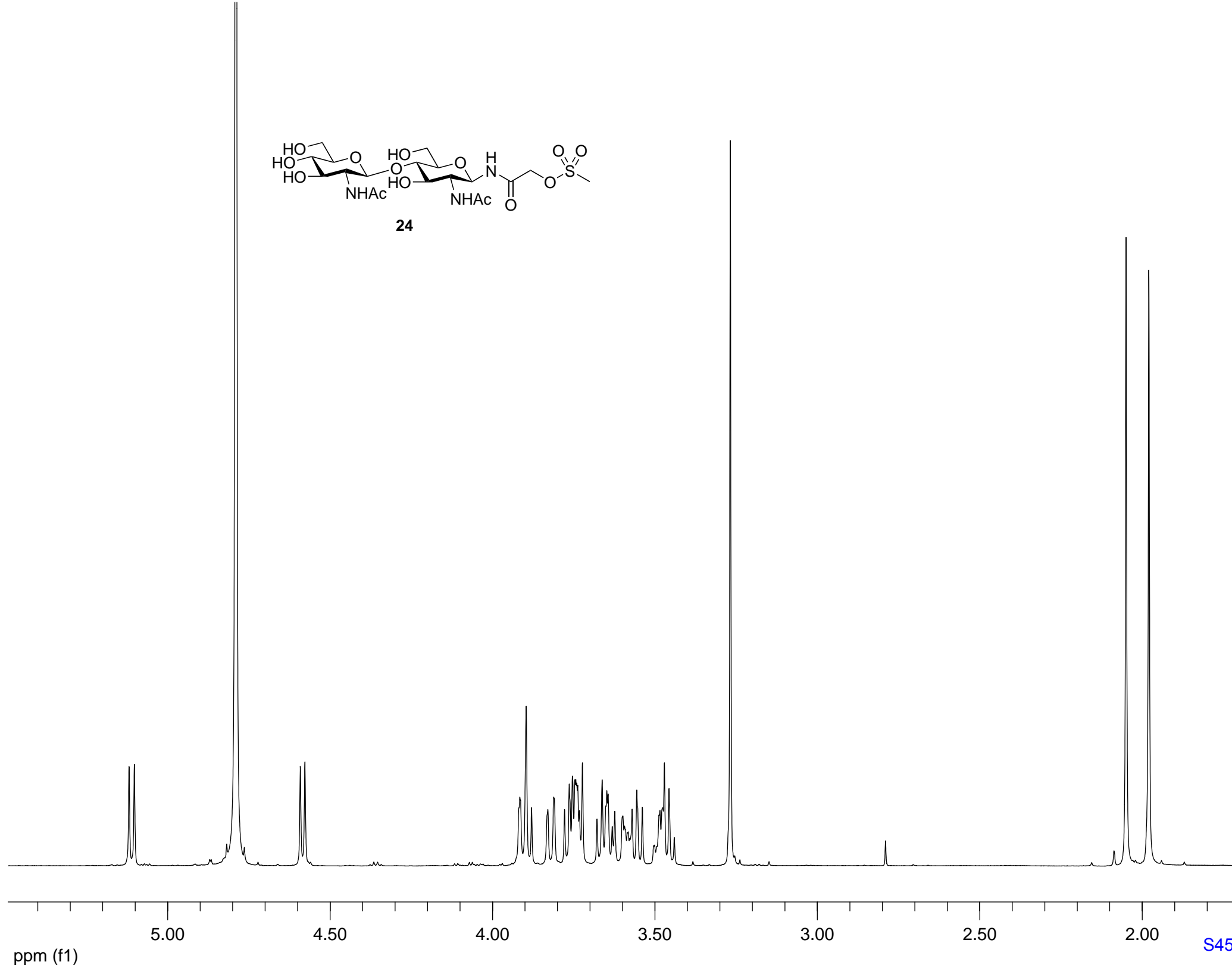
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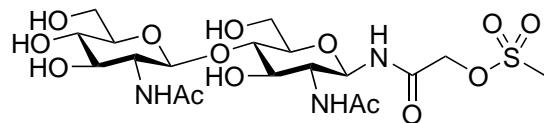




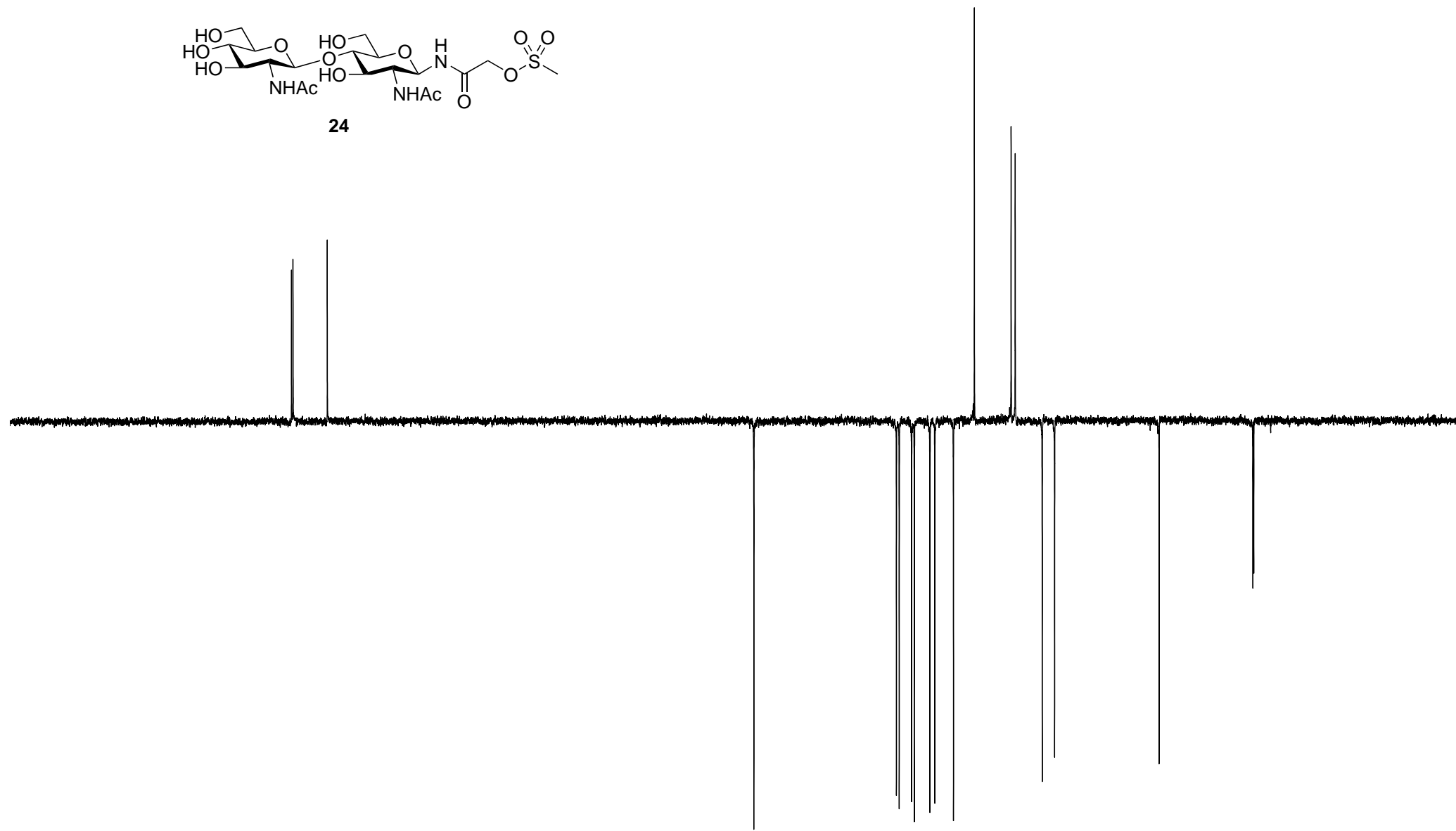


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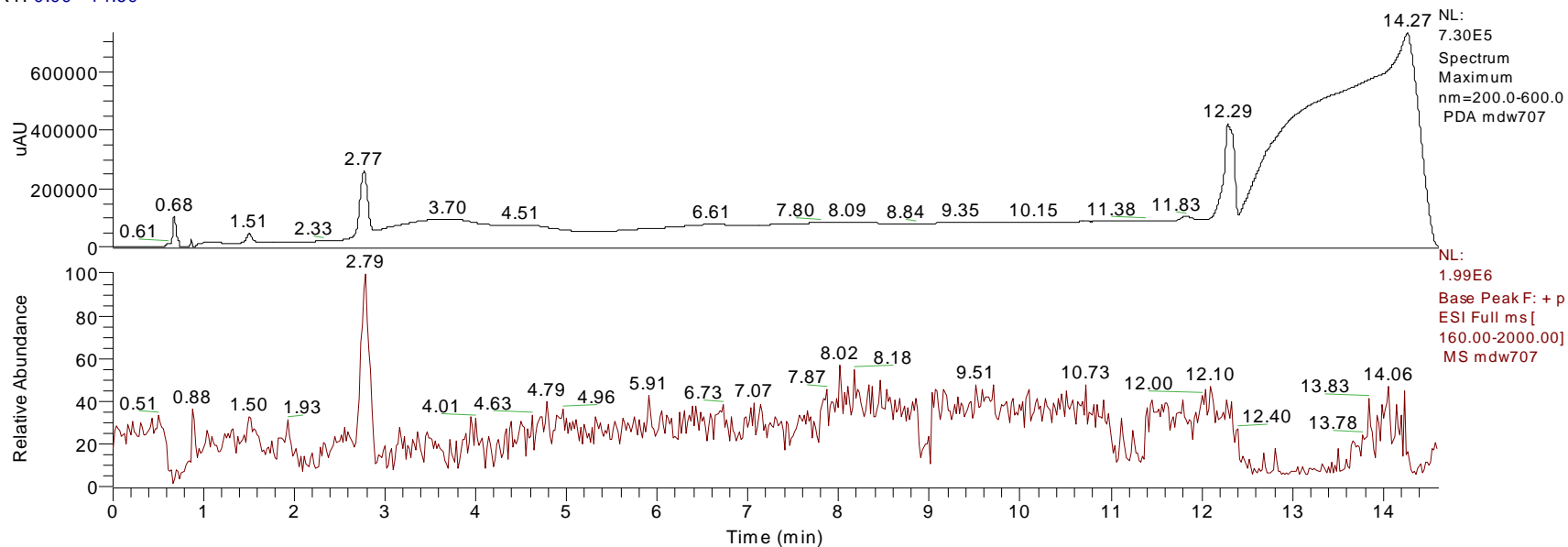




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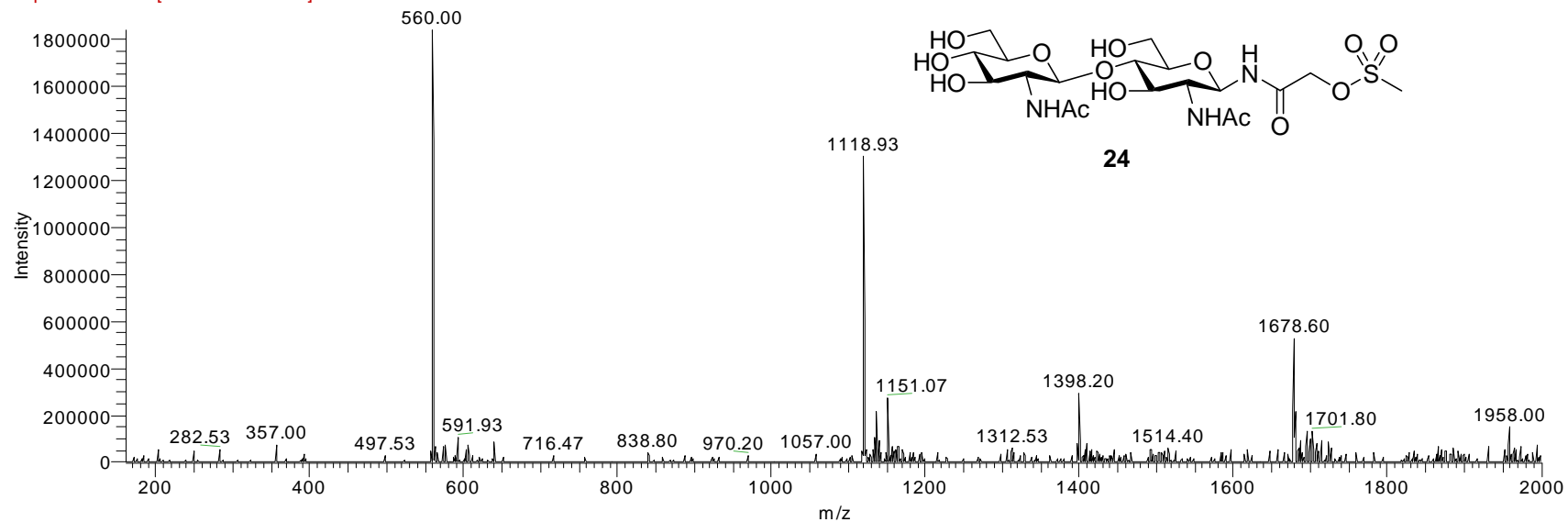


RT: 0.00 - 14.60

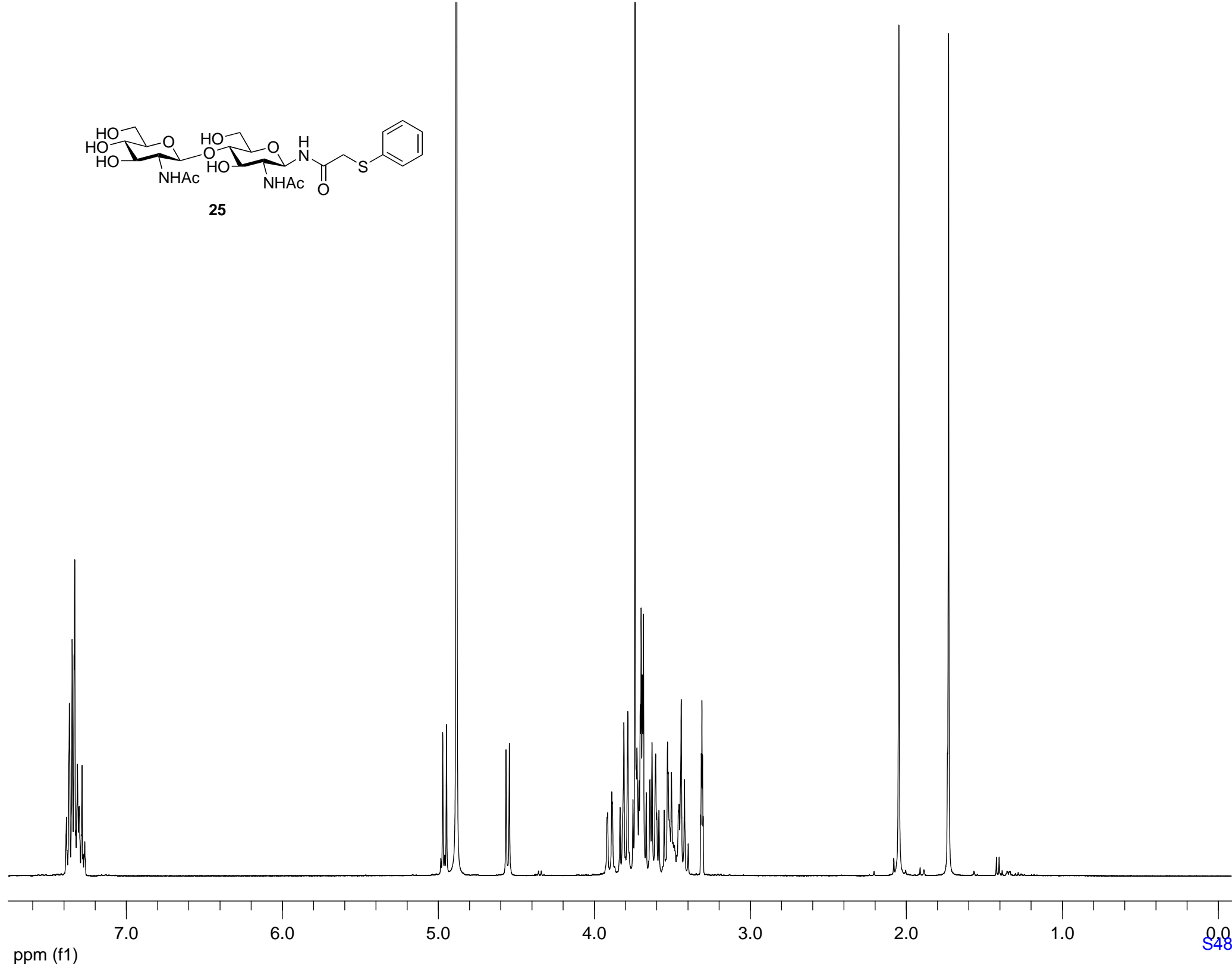
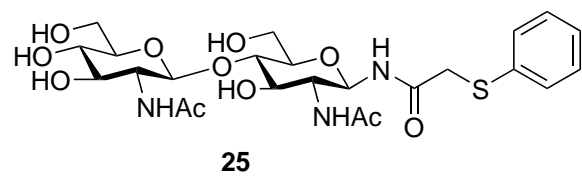


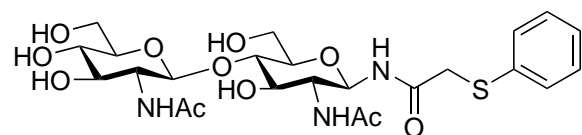
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Processing Method:  
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Injection Volume (µl): 20.00  
Sample Weight: 0.00

mdw707 #143 RT: 2.77 AV: 1 NL: 1.84E6  
F: + p ESI Full ms [ 160.00-2000.00]

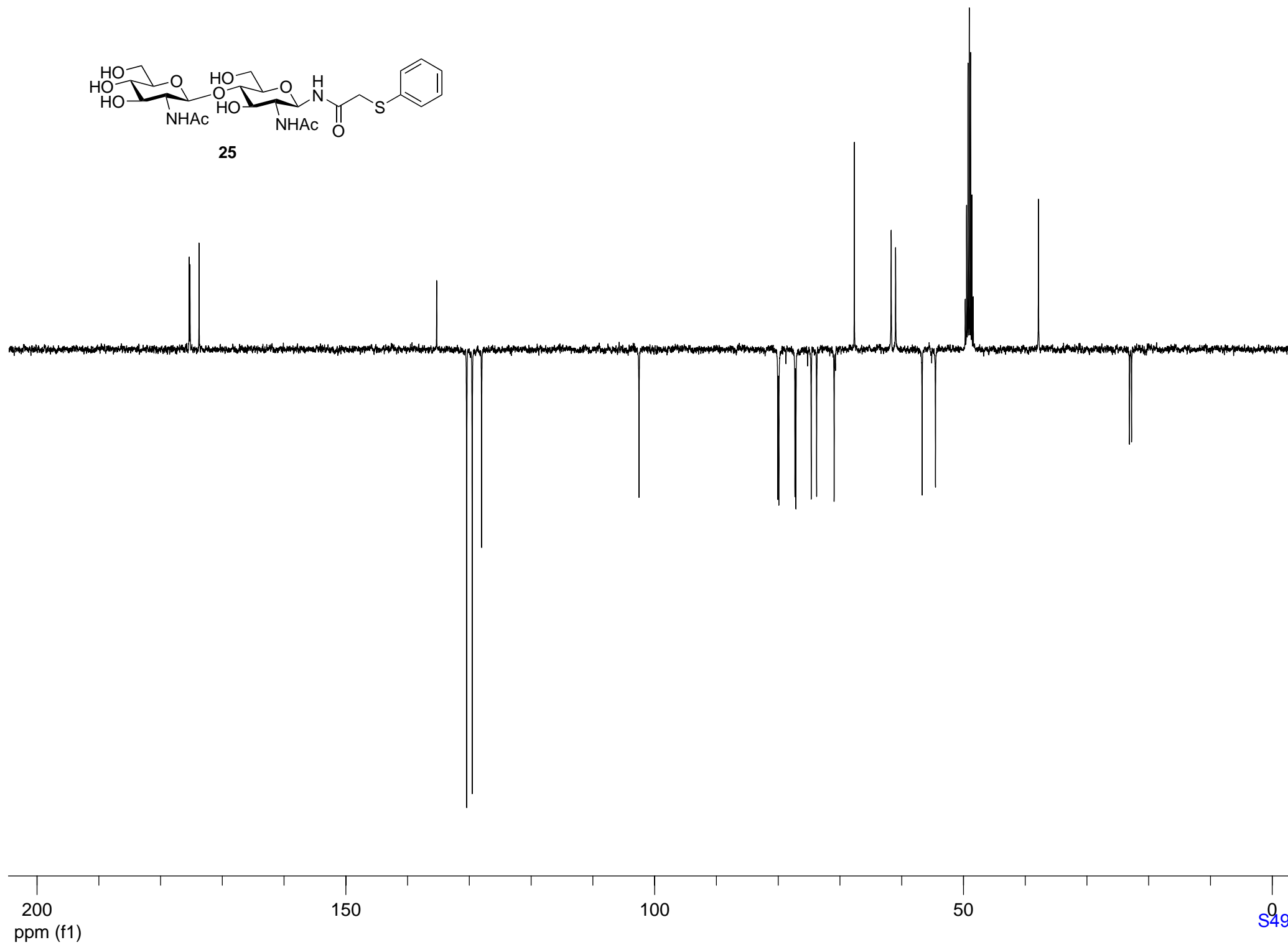




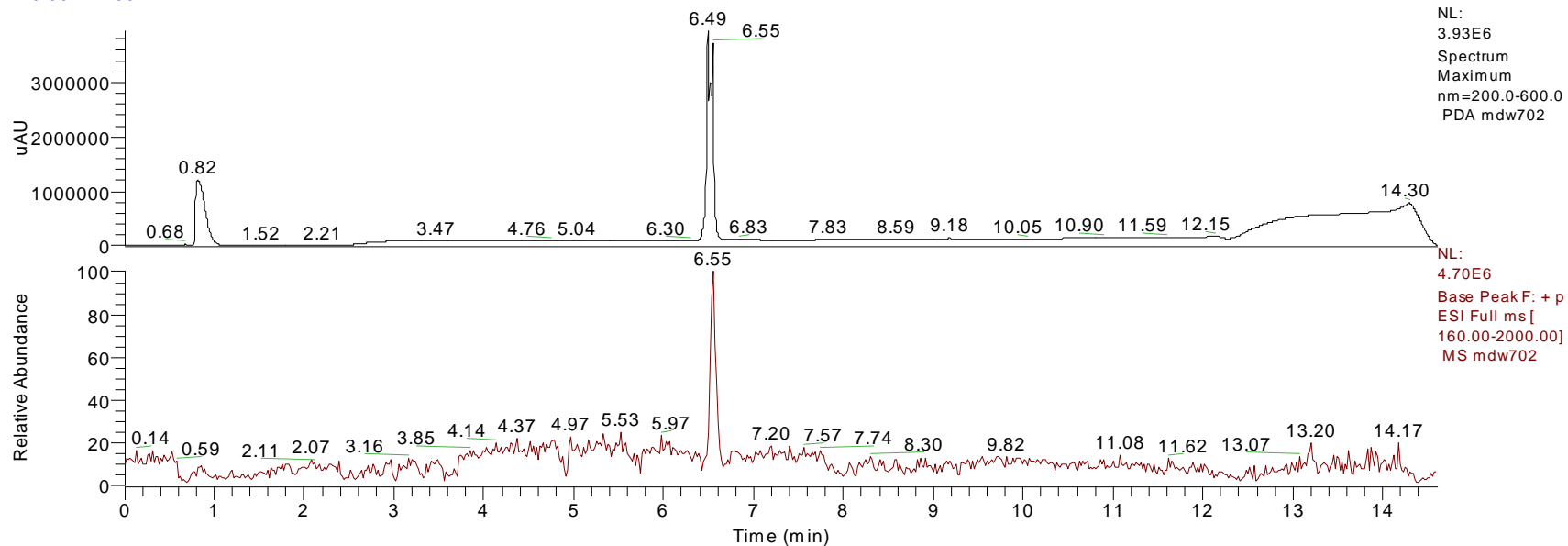




25



RT: 0.00 - 14.60



Instrument Method: C:\Xcalibur\methods\General(TFA)\0050%B00\_C=TFA\_20ul\_+pf 15min.meth  
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Injection Volume (µl): 20.00  
Sample Weight: 0.00

mdw702 #339 RT: 6.53 AV: 1 NL: 4.15E6  
F: + p ESI Full ms [ 160.00-2000.00]

